UNIVERSITÀ DEGLI STUDI DI NAPOLI
"FEDERICO II"

RESEARCH DOCTORATE

IN

APPLIED BIOLOGY

Physiology

(XXVI cycle)

Functional characterization of molecular actors of the Nitrogen Signalling Pathways involved in the control of the Symbiotic Nitrogen Fixation

Coordinator

Prof. EZIO RICCA

Candidate

LUDOVICO MARTINS ALVES

Tutor

Dott. MAURIZIO CHIURAZZI

Academic Year 2013-2014

Abstract

The nodulation process resulting from the symbiosis of legume roots and rhizobia allows legumes to be the major natural nitrogen-provider to the ecosystem, turning them into an economically viable replacement for fertilizers. The underlining regulatory mechanisms of nodule organogenesis have yet to be fully clarified. Nitrate has a role in nodule formation, either acting as a nutrient or a signal and its uptake soil is a critical process controlled by complex regulatory networks. In Arabidopsis thaliana *various members of the NPF and NRT2 families are involved in nitrate uptake and distribution. We previously reported that various orthologous NPF and NRT2 genes of* Lotus japonicus *showed a repressible, inducible or constitutive response to provision of nitrate. Biochemical characterization of one of the* Lotus japonicus *NPF family members – LjNPF4.6 was performed through a molecular genetics approach. LjNPF4.6 has a spatial profile that matches that of a nitrate transporter and its dual-affinity transport activity was confirmed through heterologous expression in* Xenopus laevis *oocytes. LjNPF4.6 is necessary for proper plant recognition and uptake of high concentrations of nitrate, as shoot and root development of knock-out plants was impaired when compared with wild-type.*

Ethylene-responsive transcription factors of the AP2/ERF family have been shown to be regulated by nitrogen status and induced during early nodulation. LjRAP2.4 is one such transcription factors, whose orthologue in Arabidopsis is involved in many ethylene-dependent processes, such as hypocotyl development, photosynthesis control and drought stress control. In legumes, ethylene has an inhibitory effect upon nodulation, so any downstream *signal of its pathway has a potential regulatory role over nodule organogenesis. Mutants overexpressing and RNAi silenced LjRAP2.4 demonstrate that LjRAP2.4 has a regulatory role in hypocotyl development in the dark and assays with the ethylene precursor ACC confirm that this role is ethylene-responsive. LjRAP2.4 is also involved in the regulation of the nodulation process, with overexpressing LjRAP2.4 plants demonstrating increased nodulation compared to wild-type.*

Keywords: Nodule organogenis, nitrate transporters, transcription factors, NPF, AP2/ERF

The work of this doctorate thesis has been developed at the Istituto di Genetica e Biofisica (IGB) "Adriano Buzzati Traverso" CNR di Napoli, CNR di Napoli, at the nitrogen-fixating symbiosis laboratory of Dr. Maurizio Chiruzarri; for his outstading scientific leadership, academic support and constant guidance that made the work you hold your hands possible, I am extremely grateful. No matter the frustrations, hardships, depressions or obstacles, the scientific method is the most powerful manifestation of the awesome human spirit. Every scratch or nudge that one can make in the limits of our knowledge, any small grain of sand one can add to the beaches of the accumulated lore of mankind, is worth any price. I also want to thank Dr. Alain Goujon and Dr Benoit Lacomb of the Institut Claude Grignon CNRS/INRA/SupAgro/UM2 in Montepellier, whose hospitality and support allowed me to complement the biochemical analysis required for this project. I will never forget the dedication of our team, and want to thank all my fellow laboratory companions that I will always hold dear: of course, the ever-patient Vlado that made everything work the way it should, the cheerful Aurora that welcomed everyone and keep us together, the dedicated Giugi whose work I was proud to follow upon, the team "big sister" and lab veteran Alexandra and our students Franscesco and Dora. And of course, my family, that was ever patient and enduring in their quiet support.

Table of Contents

1. Introduction

1.1 Nitrogen Metabolism

1.1.1 Biological Importance of Nitrogen

After carbon, hydrogen and oxygen, nitrogen is the most abundant element in living organisms. Nitrogen (N) is a fundamental element, as it is a constituent of a vast number of molecules of biochemical interest: peptides, nucleic acids, phytohormones, coenzimes, pigments, iminosugars, polyamines and many others.

On Earth, most of the nitrogen is in the air, with four fifths of this atmospheric nitrogen being presented upon the form of molecular nitrogen (N_2). However, only a relatively small number of species can convert atmospheric nitrogen into molecular forms that are useful for living organisms, and integrate this element into biological systems. The process through which interdependent organisms interact with each other and cooperate to convert and reutilize bioavailable nitrogen into different molecular forms is called the Nitrogen Cycle (Fig. 1)

This N cycle starts with **fixation**, in which nitrogen-fixing bacteria reduce atmospheric nitrogen in order to produce ammonia (NH_3 or NH_4^+) that might be used by the majority of living organisms. Ground bacteria obtain energy through oxidation of ammonia to nitrite (NO_2^-) and nitrite to nitrate (NO_3^-); this process is called **nitrification**. At this point of the cycle, plants and many bacteria can easily take up and reduce nitrate and nitrite through action of the enzymes nitrate reductase (NR) and nitrite reductase (NiR). In the particular case of plants, they reduce the nitrate to nitrite in the cytoplasm through the nitrate reductase, transport the nitrite to the chloroplasts and there, through the action of nitrite reductase, reduce nitrite to ammonia. This ammonia can then be integrated into amino acids of the plants and other nitrogen-containing biomolecules; animals obtain nitrogen-compounds when consuming plants, using their essential and non-essential amino acids for protein synthesis. When an organism dies, microbial degradation of the proteins returns ammonia to the soil, where it is converted to nitrite and nitrate to fulfill the energetic requirements of soil bacteria; these bacteria are also responsible for keeping a balance

between fixated nitrogen and atmospheric nitrogen by converting nitrate to molecular nitrogen in anaerobic conditions, through a process called **denitrification**.

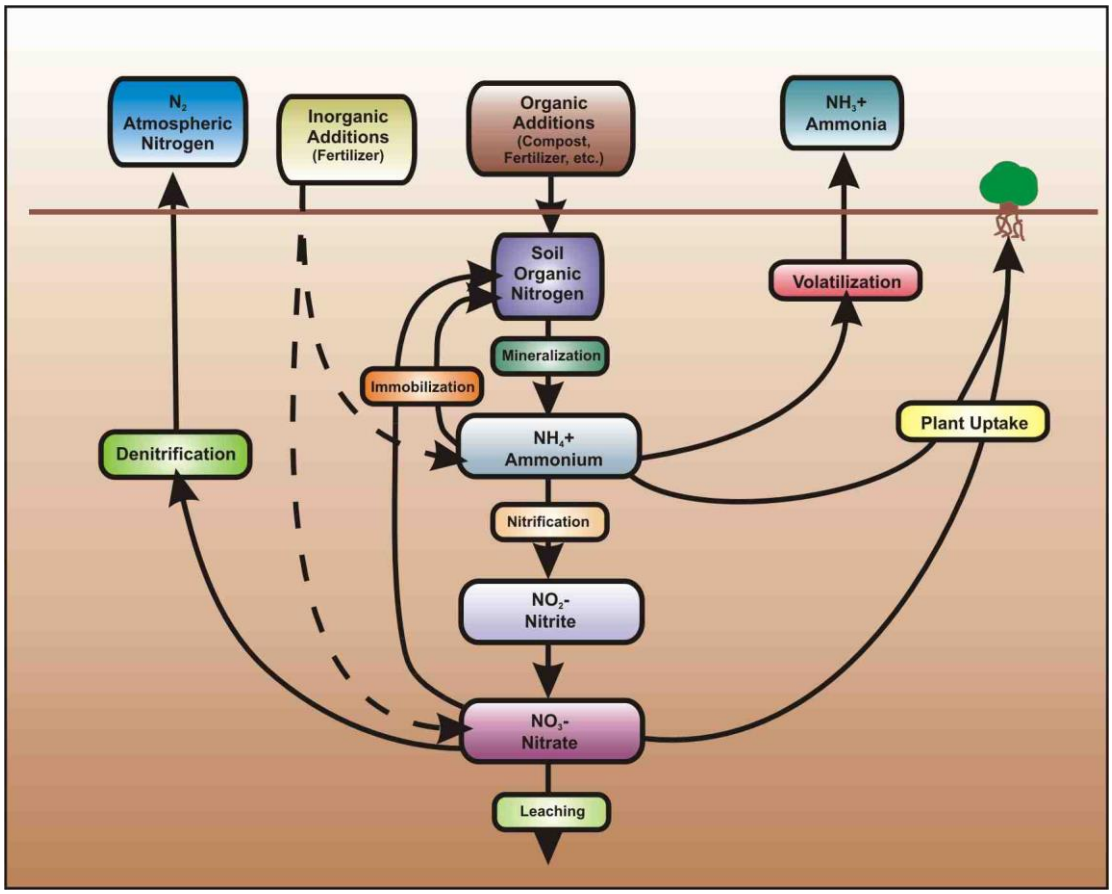


Fig. 1: Schematic representation of the Nitrogen Cycle. (Nelson and Cox 2008)

1.1.2 Molecular Nitrogen (N_2) Fixation

As it was previously stated, only a relative few microorganisms can fix atmospheric nitrogen. Amongst these are cyanobacteria, who live in the soil and both seawater and freshwater; other bacteria like the genus *Azotobacter*; and nitrogen-bacteria that fix nitrogen and live as symbionts in nodules present in the roots of leguminous plants. The first relevant product of nitrogen fixation in all these organisms is ammonia, which can be used by other organisms, either immediately after transformation or after being transformed into other soluble products like nitrite, nitrate or already integrated into amino acids. Molecular nitrogen is reduced to ammonia through the following exoenergetic reaction:

$$N_2 + 3H_2 \rightarrow 2NH_3$$

The triple bound between N≡N is very stable, which means that nitrogen fixation has a very high energy of activation and molecular atmospheric nitrogen is practically inert in normal conditions. This barrier must be overcome, at least in part, through the hydrolysis of ATP. As such, the complex reaction is:

$$N_2 + 10\,H^+ + 8e^- + 16\,ATP \rightarrow 2NH_4{}^+ + 16\,ADP + 16\,P_i + H_2$$

The biological nitrogen fixation is catalyzed by a protein complex denominated nitrogenase complex, which includes two components essential for this process: dinitrogenase reductase and dinitrogenase (Fig. 2). The dinitrogenase reductase is a dimer with identic subunits and can be oxidized or reduced by an electron; it also contains two ATP/ADP binding sites. Di-nitrogenase is a tetramer with two copies of different sub-units.

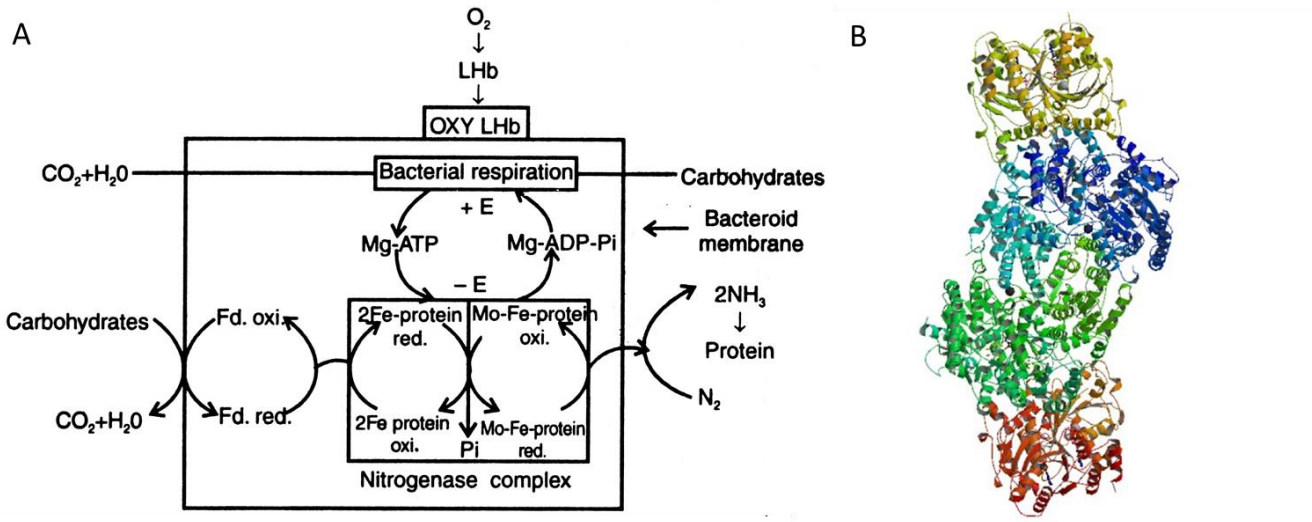
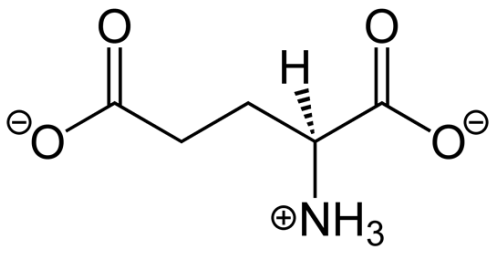


Fig. 2: Nitrogenase enzyme binding complex and N_2 fixation (A): The two main components of the complex, nitrogenase (MoFe protein) interacts with Mg^{2+} and ATP, and nitrogenase reductase (Fe protein) catalyzes the N_2 to NH_3 reduction. The reduced ferredoxin (Fd. red) provides electrons (e-) to Fe protein, which then reduces Mo-Fe-protein, with release of Pi, from the MgATP produced by bacterial respiration. In the case of the nodules, leghaemoglobin binds with oxygen, forming oxyleghaemoglobin and maintaining oxygen concentration low. B) Representation of the nitrogenase enzyme complex, with the MoFe protein at blue and green in the center and the Fe protein dimers in the opposite ends of the complex, at yellow and red.

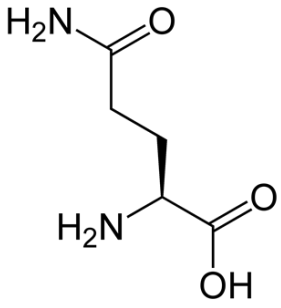
Dinitrogenase is reduced through the transfer of electrons from di-nitrogenase reductase; di-nitrogenase has two binding sites for the reductase and the eight electrons that are required are transferred in a single step. During this process, the reductase enzyme hydrolyzes ATPs. Reduced ferredoxin is usually the source for the electrons required for the di-nitrogenase reductase; in turn, ferredoxin obtains its electrons from pyruvate.

The nitrogenase enzyme complex is extremely vulnerable to oxygen, requiring anaerobic conditions in addition to the intensive energetic costs involved in these reactions. These two factors greatly limit the number of organisms that integrate their own nitrogen into biomolecules; nitrogen-fixating organisms take different approaches to deal with these requirements. Anaerobic bacteria fix nitrogen only in anaerobic conditions; aerobic bacteria like *Azotobacter vinelandii*, have a partial compartmentalization of electron transport apart from ATP synthesis, assuring that oxygen is used for oxidative phosphorylation at the same speed as it enters into the cell, preventing build-up of oxygen. Cyanobacteria have yet another solution: one out of each nine cells becomes an heterocyst, a cell specialized in nitrogen-fixation that possesses three additional cell walls – including a glycolipid one that forms a hydrophobic barrier to oxygen – and loses the photosystem II and the capacity to realize photosynthesis, depending now from the neighboring vegetative cells to provide it with carbohydrates.

As previously mentioned, reduced nitrogen is integrated in biological systems first as ammonia, followed by amino acids and later in other nitrogen-containing biomolecules. The entry point for nitrogen into various biochemical cycles and pathways are the two amino acids glutamine (Gln, Q) and glutamate (Glu, E).



Glutamate – Glu/E



Glutamine – Gln/Q

These two amino acids play an essential role in the catabolism of amino acids; in fact, the majority of amino acids are obtained from glutamate through transamination reactions,

while the amide group of glutamine is the source of the amide groups of various biosynthetic pathways. Due to the later relevance of glutamine and glutamate, the biosynthetic pathways that produce glutamate and glutamine for integration and assimilation of ammonia into biosystems, are simple and simple across all life forms.

The main pathways are:

- GDH/GS; in organisms with abundant nitrogen sources
- GS/GOGAT; in the majority of soil bacteria and plants

In the GDH/GS pathway, the enzymes glutamate desidrogenase (GDH) and glutamine synthetase (GS) are involved; on the GS/GOGAT pathway, glutamine synthetase (GS) and glutamate synthetase/glutamine oxoglutarate aminotransferase (GOGAT) are involved. While the GDH/GS pathway is the most energetically efficient, the GDH enzyme (ubiquitous across the organism) has a low affinity for ammonium (Km ≈ 1mM), which means that this pathway is only used under high ammonium ion concentrations. The GDH reaction is as follows: gs

$$\alpha\text{-}ketoglutarate + NH_4{}^+ + NADPH \rightarrow L - glutamate + NADP^+ + H_2O$$

The most common pathway, GS/GOGAT, is based upon two reactions: first, glutamate and the ammonium ion react to form glutamine, a reaction catalyzed by GS. This is a two steps reaction with one intermediary – γ-glutamylphosfate – which remains bound to the GS enzyme.

$$\textbf{Glutamate} + NH_4{}^+ + ATP \rightarrow Glutamine + ADP + P_i + H^+$$

The second reaction assumes the intervention of glutamate synthetase that catalyzes the reductive amination of α–ketoglurate, an intermediary of the citric acid cycle, using glutamine as donor of an amine group to regenerate glutamate.

$$\alpha\text{-}ketoglutarate + glutamate + NADPH + H^+ \rightarrow 2\ glutamate + NADP^+$$

The glutamine synthetase is present in every organism, while glutamate synthetase is not present in animals. Besides being important for ammonia assimilation, this reaction is also a central point for amino acids metabolism; in fact, this is the main pathway for removing toxic ammonia that accumulates, converting it to a non-toxic compound – glutamine – which can be transported in the bloodstream. Glutamine synthetase is highly-regulated in all organisms, which is not surprising, if one considers its crucial role as entry point for reduced nitrogen into cellular metabolism.

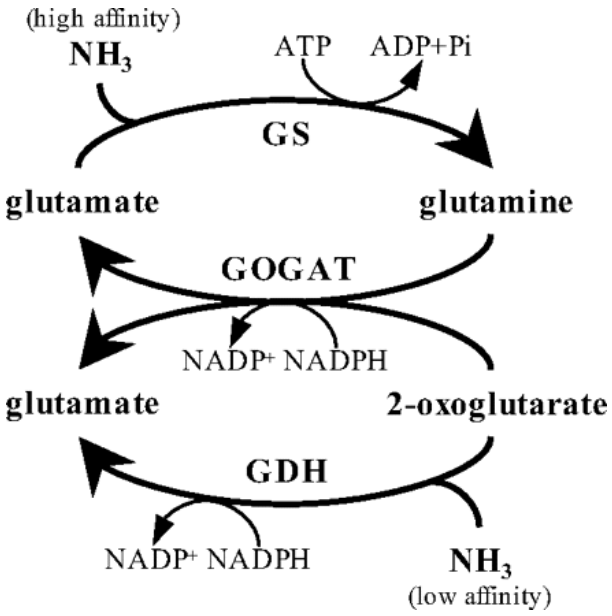


Fig. 3: Glutamate/Glutamine Integration. The three-enzyme circuit assimilates NH_4^+ and produces two central intermediates, glutamine and glutamate. GS catalyzes glutamine synthesis. Glutamate can be synthesized by the action of either GS/GOGAT or GDH, respectively, with high or low NH_4^+ affinity. The two glutamate molecules have no known functional differences (Yan 2007).

1.2 Symbiosis and nitrogen fixation: nodules

The nitrogen-enrichment of soils containing leguminous plants is a testament to the efficiency of this symbiosis; such fact allows for agronomical methods like culture rotation (in which plants that consume soil nitrogen are alternated with leguminous plants that replenish the soil nitrogen) or green manure (burying special cultures of leguminous plants with the objective to increase nitrogen levels and fertilize the terrain).

The intensive energetic requirements and the needed anaerobic conditions have probably been determinant for evolution to favor association between higher plants and bacteria. The bacteria present in the nodules of plant roots have access to a great energetic reserve, the carbohydrates and intermediaries of the citric acid cycle available in the plant. It has been demonstrated that about 20% of the photosynthate must be allocated to the nodules to allow the occurring of the nitrogen fixation symbiotic pathway. Thanks to this readily available energy source, the bacteria of the nodule in roots can fix nitrogen at a speed hundreds of times faster than their independent counterparts that inhabit the soil. To address the problem of the oxygen's toxicity, the bacteria from the root nodules are immersed in a solution of an oxygen-binding protein, leghemoglobin (mM range into the cytoplasm of nodular infected cells), produced by the plants partners, with the heme group supplied mainly by the bacteria. Leghemoglobin binding with all the oxygen present in the nodule (Km = 0.01 µM), prevents it from interfering with the nitrogenase enzyme complex and at the same time provides oxygen to bacteria for respiration.

1.2.1 Nodule organogenesis, structural organization and metabolism

The establishment of symbiosis between legumes and rhizobia involves the activation of genes in both the host and the bacterial symbiont. The formation of a nodule requires the reprogramming of differentiated root cells to form a primordium, from which a nodule can develop. Furthermore, the bacteria must infect the root before the nitrogen-fixing root nodule can be formed. These steps in nodule formation involve changes in three root tissues, namely epidermis, cortex, and pericycle. These morphological changes are preceded by the induction of certain genes (nodulins) in the broad region of the epidermis, such as the early-nodulin genes ENOD12 and ENOD11. Upon inoculation with rhizobia, root hairs will deform. This is caused by re-initiation of tip growth in these cells, but with a changed growth direction; these morphological changes are also preceded by modification of the actin skeleton. In some root hairs, the rhizobium-induced deformation leads to a root hair to curling; this is probably due to a gradual and constant reorientation of the growth direction of the root hair, until a 360º curl is reached. During the curling, bacteria become entrapped in the pocket of the curl, where the plant cell wall is modified at the local level, the plasma membrane invaginates and new plant material is deposited, forming a tube-like structure -

the infection thread. The infection thread contains the bacteria, and will grow towards the base of the root hair cell and subsequently to the nodule primordium. A rhizobium-secreted signal (see below) triggers cells response in both cortical and pericycle cells even before the infection thread crosses the epidermis. Gene activation takes place at the same time and for example the early nodulin ENOD40 is induced in the pericycle, and both these and cortical cells re-enter in the cell cycle and undergo various cell divisions. Nodule vascular structures are arisen from pericycle cells division, whereas cortical cells divisions (outer or inner cortex; see below) lead to the nodule primordium formation (Geurts and Bisseling 2002; Dixon and Kahn 2004).

The transition from a nodule primordium to a functional young nodule occurs after infection of primordial cells. The cells at the base of the primordium establish a radial pattern of central tissue surrounded by perpipheral tissues. Cells at the apex of the primordium form a meristem, which, by division, maintains itself and adds new cells to the different tissues according to and adds new cells to the different tissues according to the pattern established at the base of the primordium. Meristematic and primordium cells have different identities, with different activated genes and meristematic cells never being infected by rhizobia (Geurts and Bisseling 2002).

The rhizobia's signals that are essential to trigger plant response are called Nod factors. The nodulation genes of rhizobia are induced upon sensing certain plant-specific flavonoid molecules, secreted by plant root; flavonoids trigger the bacterial transcriptional regulator NodD, which in turn activate the other nodulation factors. The basic structure of Nod factors produced by different rhizobial species is very similar. Generally, they consist of a β-1,3-linked N-acetyl-β-glucosamine backbone with 4 or 5 residues of which the non-reducing terminal residue is substituted at the C2 position with an acyl chain. Depending on the rhizobial species, the structure of the acyl chain can vary, and substitutions at the reducing and non-reducing terminal glucosamine residues can be present (Cullimore et al. 2001). Different rhizobium strains might synthetize different Nod factors carrying different substituting groups at the non-reducing end; these features coupled to the different types of flavonoids triggering molecules (e.g. naringenin, luteolin, quercetin) secreted by legume roots determine the molecular bases of host-specificity in the legume/rhizobium interaction (Fig. 4).

Two types of nodules can be found in the roots of legumes: the undetermined nodules that form in the roots of temperate legumes like *Pisum*, *Vicia* and *Medicago*; and the determined nodules that are formed in the roots of tropical legumes like soybean, *Lotus*, *Phaseolus* and *Lupinus* (Fig. 5).

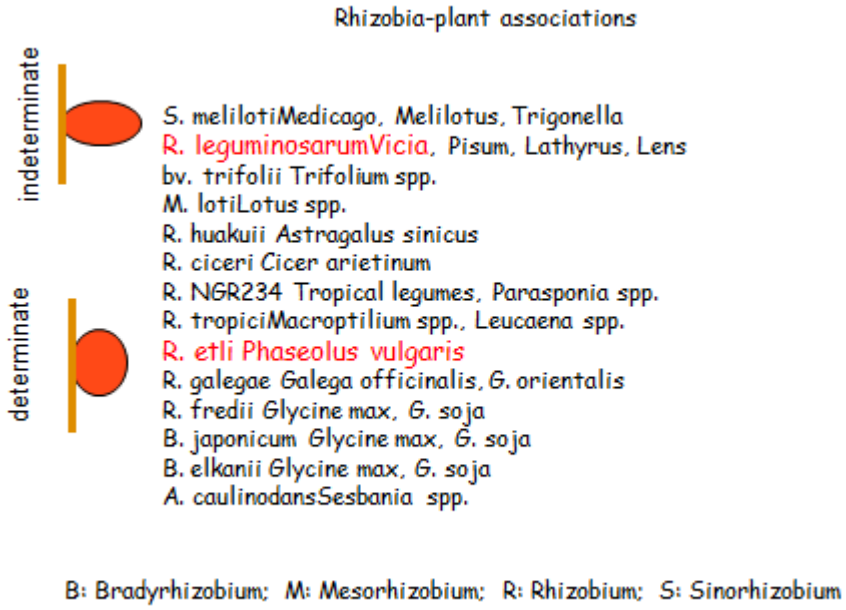


Fig. 4: Species-specificity of the legumes-rhizobia interactions. Legume species with their rhizobia symbiotic partner and the type of nodule they form are indicated.

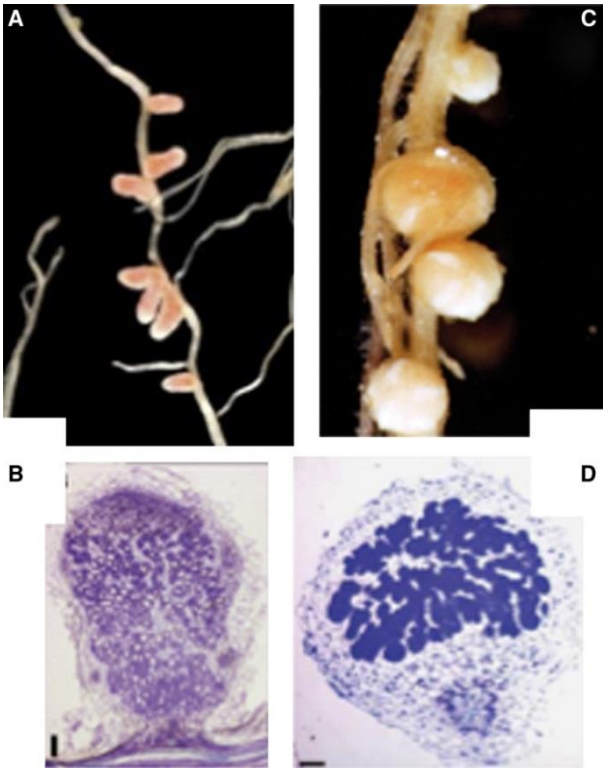


Fig. 5: Two types of nodules in legumes. Upon inoculation with their respective rhizobial symbionts, Medicago develop elongated nodules (A) of the indeterminate type (B), while *Lotus japonicus* develop spherical nodules (C) of the determinate type (D). In panels B and D are shown semithin nodule sections embedded in Historesin and stained with 1% basic toluidine blue for light microscopy (Desbrosses and Stougaard 2011).

Undetermined nodules are characterized by the presence of an apical region formed from undifferentiated cells, namely, a persistent apical meristem. The root cells that re-enter the cell cycle and divide after infection are those in the internal layers of the cortex. These form both the nodule primordium and later the central tissue of the mature nodule. While the meristematic cells are pushed to the outside, the infection tube – in which rhizobia proliferate –actively penetrates the nodule primordium, forming ramifications and inverting the growth direction in order to develop towards the apical nodule meristem. In longitudinal sections of undetermined nodules, cytological and molecular criteria allow to spatially define the various tissue zones of nodule maturity: bacteria-free zone I (apical meristem); the zone II (invasion), in which the infection tube infects new cells in division; the zone III (early symbiotic zone) where invaded cells are filled with mature nitrogen-fixating bacterioids; the zone IV (late symbiotic zone) with invaded cells filled with senescent bacteroids. The interzone II-III, located between the invasion zone and the symbiotic zone, is characterized by accumulation of amide in the amyloplasts on invaded cells and important events of modification of gene expression. It has been observed in this zone the expression of specific nodulins which confirm the existence of a spatial-defined program of gene expression. Therefore, the presence of a persistent apical meristem that constantly adds new invading cells to the central tissue characterizes undetermined nodules, in which nodule growth and function are contemporary events (Vasse et al. 1990; Battisti et al. 1992).

In the determined root nodules the presence of a persistent meristem is not evident. While the development program of the determined nodule has not been entirely clarified, they are described as globular structure formed by peripheral tissue and central tissue, in which the cells present a certain level of developmental synchronization (Bisseling et al. 1980; Bisseling et al. 1983). This model predicts the simultaneous invasion of some cells of nodule primordium, which undergo a few divisions and differentiate, increasing their volume. Growth of a determined nodule seems to be mainly due to cellular expansion, partially due to mitotic activity and there cannot be found any zones of development of central tissue that are analogous to those of undetermined nodules. Recent reports state

that in the determined nodules have been found defined zones that have differences in terms of development, namely, at the point in which they are invaded by the infection tread and different profiles of gene expression (Patriarca et al. 2004).

In independent conditions, bacteria only assimilate the nitrogen available in the soil in the form of nitrate, ammonium, amino acids and other biomolecules; when they have invaded the cytoplasm of nodule cells, bacteroid diversify their metabolism, differentiating and fixating atmospheric nitrogen, reducing it to ammonium which is then integrated into amino acids and exported to the plant. The bacteria shift from an assimilating nitrogen metabolism, which they maintain during the formation of the infection thread, towards a fixing nitrogen metabolism, upon reaching the inside of the symbiosome. According to this shift, the genes of bacteria involved in the transport and assimilation of nitrogen are down-regulated at the moment of endocytosis into the cells of the nodule primordium.

1.2.2 Bacteria partners and nodule organogenesis

Various species of bacteria belonging to the α-proteobacteria class and the Rhizobiales order can engage in a symbiosis with plants of the leguminous family. These bacteria, which, based on this symbiotic behavior, are collectively called rhizobia, are a very diverse group divided into into four different families, the Rhizobiaceae, the Phyllobacteriaceae, the Hyphomicrobiaceae, and the Bradyrhizobiaceae. Within these families, the following genera have capacity to establish nitrogen-fixing symbiosis with leguminous plants: *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Allorhizobium* (Spaink 2000).

Even with this genetic diversity, rhizobia genera have many common genetic and biochemical characteristics related to their symbiosis-capacity: they can recognize specific signal molecules, special structures and regulatory molecule, thus allowing them to adapt to the different condition in the plant host and bypass defense response from the host. As cited above, rhizobia are extraordinarily selective about their plant partners, and they are not able to establish symbiosis and develop functional nodules with other legumes. For example, *L. japonicus* can develop fully functional nitrogen fixing nodules with M. loti and ineffective or partially effective nodules with NGR234, *Rhizobium etli* and *Bradyrhizobium* spp. This specificity is assured through recognition at the molecular level, in the basis of a two-way

signal exchange between rhizobia and plant. Rhizobial NodD proteins mediate host recognition by interacting with specific flavonoids or isoflavonoids secreted by the host plant roots, triggering transcription of bacterial Nod-factors, signals that are then detected by the host. The dynamism of this signaling mechanism was demonstrated by using two bacteria, *R. etli* and *M. loti*, from different cross-inoculation groups nodulating bean and Lotus, respectively. Both strains synthesize Nod-factors with the same structure. An artificially bypassing of the flavonoid NodD activation by expression of a constitutive nodD transcriptional activator (FITA) in these rhizobial strains was sufficient to extend their host range beyond their cross-inoculation group to encompass both bean and Lotus corniculatus. (Spaink et al. 1991; Cardenas et al. 1995; Banba et al. 2001)

L. japonicus is not able to achieve functional symbiosis than with any rhizobia other than M. loti. Even with *Bradyrhizobium loti*, morphologically normal nodules are formed in the roots but are unable to reduce atmospheric nitrogen (Nod + Fix - phenotype). The ability to reduce nitrogen is also lacking upon infection with *Rhizobium etli* (the symbiotic partner of *Phaseolus vulgaris*) due to the lack of cell invasion of the cortical region, resulting in rapid senescence of nodules (Banba et al. 2001). The sequencing of *Mesorhizobium loti* was completed in 2000 has showing that the genome is made up of a single chromosome (7036.074 bp) and two plasmids, respectively denominated PMLA (351.911 bp) and pMLb (208.315 bp). The genes for nodulation and nitrogen fixation are located on the bacterial chromosome. The strains of *Mesorhizobium loti* commonly used for research are: R7A, NZP2235, JRL501, MAF303099 and TONE. Most of these strains were engineered with reporter genes such as GFP or lacZ, constitutively cast, to allow the analysis of the infection from the first moments of the induction until the development of the nodule symbiosis.

1.2.3 Early steps of nodule organogenesis

The released nodulation factors are recognized by Nod factor binding proteins, such as NFBS1 and NFBS2 and lectines. One of the first responses to Nod factors in the plant is the depolarization of the plasma membrane, caused by a very rapid calcium influx which is later followed by a potassium flux that repolarizes the membrane; then a calcium spiking, characterized by a transient and regular oscillation of the cytoplasmic calcium concentration, provides the transduction of the nod-induced signal through the root hair.

In *Lotus japonicus*, various mutants have been isolated that lack the capacity to nodulate after infection with rhizobia; these are discriminated according to their phenotype and relative position in the Nod factor signal transduction. The mutants LjNFR1 (Nod factor Reception) and LjNFR5 have a role in the primary reception of Nod factors, and as such fail to show any early nodulation phenotype (Fig. 6); this puts LjNFR1 and LjNFR5 high in the signaling cascade of Nod factor transduction. LjNFR1 and LjNFR5 code a kinase-like membrane receptor, with an extracellular domain rich in lysine. These domains have been identified in bacteria, where they form specific domains involved in binding to N-Acetylglucosamine polymers, which are the skeleton of Nod factor structure.

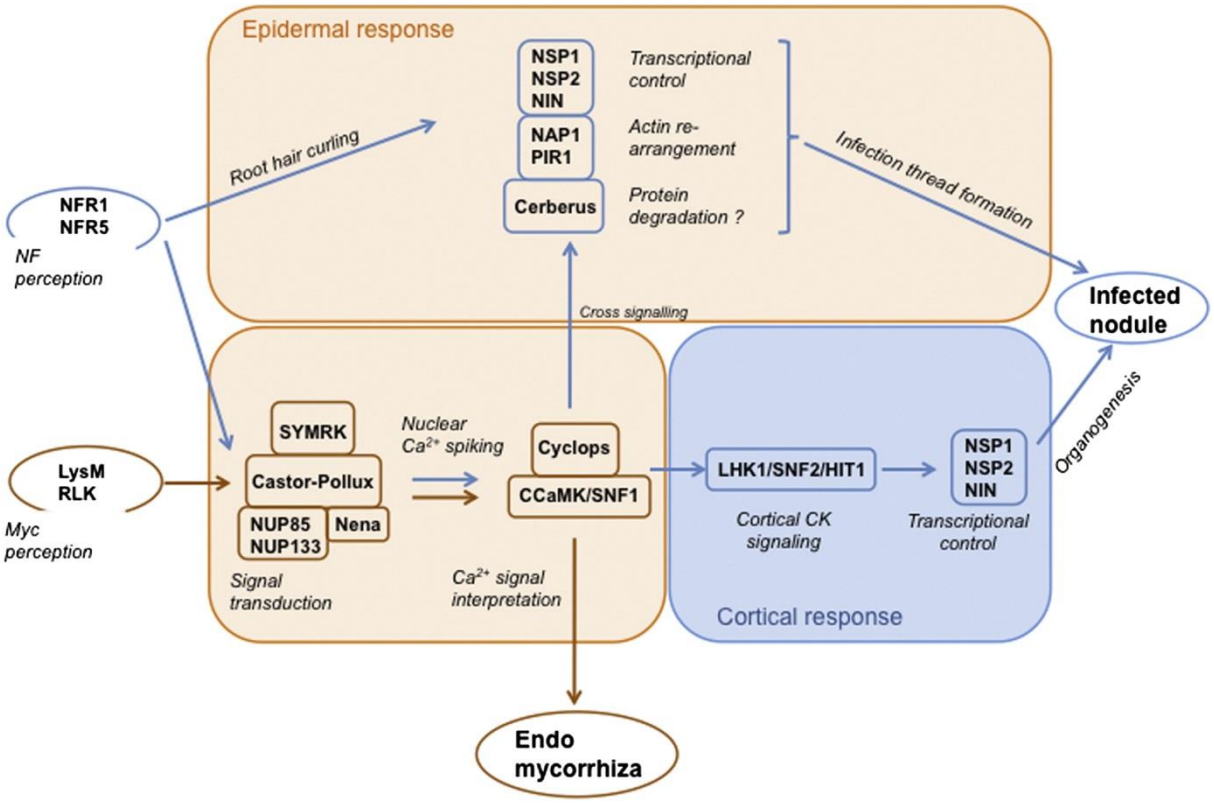


Fig. 6: *Lotus japonicus* pathways involved in nodule formation. Perception of Nod factor (NF) by NFR1 and NFR5 receptors at the plasma membrane of the epidermis triggers two parallel pathways facilitating infection thread initiation and root nodule organogenesis, respectively. The blue color indicates a different region of the root tissue represented by the cortex where nodule organogenesis takes place after signal transduction. In the figure is also indicated the Myc signal transduction involving the common signaling pathway (lower orange figure). Adapted from: (Madsen et al. 2010; Desbrosses and Stougaard 2011).

In *Pisum sativum*, PsSYM10 and in *Medicago truncatula*, MtNFP, were found to be orthologes of LjNFR5; also in Medicago, two genes, MtLYK3 and MtLYK4, have been found to code a protein with lysine-rich domains with many similarities with LjNFR1. According to the currently accepted model, the heterodimers composed by the genes LjNFR1 and LjNFR5, which probably interact through their cytoplasmatic kinase domains, represents the system of Nod factors perception, even if a direct physical interaction between these factors and the receptors has not been demonstrated. Another mutant identified in *Lotus japonicus* was SYMRK (Symbiosis Receptor Like Kinase), which also codifies a membrane kinase receptor; its phenotype puts it below LjNFR1 and LjNFR5, as the *symrk* mutant is able to perform the initial calcium influx but not the calcium spiking and curling of root hair (Fig. 7) (Sandal et al. 2002; Stracke et al. 2002). SYMRK is constituted by a signal peptide, an extracellular domain with leucine-rich repetitions, a transmembrane domain and an intracellular protein kinase domain.

A phenotype similar to SYMRK has been demonstrated in the mutants *Castor*, *Pollux* and NUP133, which, like SYMRK are co-involved into establishing the event of the perinuclear calcium spiking (Fig. 7). These genes are also involved in the mycorrhizal symbiosis pathway, suggesting that the processes of nodulation and mycorrhization have co-evolved, sharing part of their pathways and molecular actors. Castor and Pollux code potential multimeric ionic channels located in the nuclear membrane of root hairs, while NUP133 codes a nucleoporin protein. The ionic channel identified by Castor and Pollux is a cation channel with a weak preference for potassium and hence it is not directly involved in the influx/efflux of calcium ions responsible for the calcium spiking but participates to the modulation of the required nuclear envelope membrane potential (Charpentier et al. 2008).

The mutant of the CcaMK gene identifies a physiological link between the event of calcium spiking and the successive activation of nodulins and formation of nodule primordium. CcaMK codes a calcium-binding calmodulin-binding kinase protein; CcaMK acts downstream of the calcium spiking event (Fig. 7) and seems to be involved in decoding this signal, transmitting the signal through kinase activity to various primary transcriptional activators responsible for the transcription of nodulin precursors. According to this model, the NSP1 and NSP2 mutants identify two typical primary transcriptional factors of the GRAS family which co-localize in the nucleus with CcaMK and have a phenotype similar to *ccamk*;

they are able to perform calcium spiking but there is no induction of cell division in the cortex. The importance of CcaMK in this signaling pathway has been further demonstrated in mutants of *L. japonicus* with a partial, constitutive phosphorylase activity, which presents a phenotype of spontaneous nodulation even in the absence of Rhizobium and Nod factors (Tirichine et al. 2006). More recently another component of the signaling cascade has been identified with CYCLOPS, a protein carrying a coiled-coil domain and physically interacting with CcaMK in the nucleus. CYCLOPS can be phosphorylated by CcaMK and probably coadiuvate with this in the signal transduction pathway required for infection, whereas is not involved in nodule organogenesis (Yano et al. 2008)The *Lotus* gene *LjNIN* and its Pisum orthologe PsSYM35 also have a fundamental role in the induction of cell division and formation of the root nodule, as indicated by the phenotype of the *nin* mutant. NIN is a transmembrane protein with a potential signaling role, a nuclear localization and a DNA-binding domain (Schauser et al. 1999). The phenotype of the *nin* mutant is characterized by hyper-deformation of root hair and no cortical cell divisions and neither infection thread formation (Fig. 7).

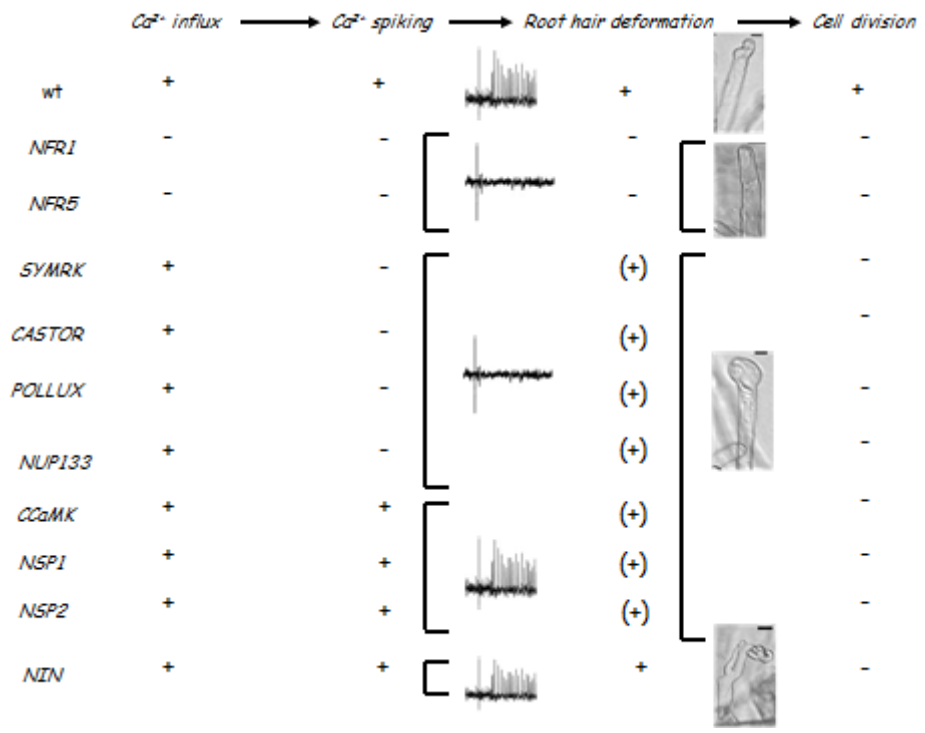


Fig. 7: Temporal cascade of events induced by Nod factors transduction pathway. Specific mutants and their related phenotypes identifying the different steps of the signaling pathway are indicated.

1.2.4 Regulation of Nodulation

Being such a complex and energy-demanding process, nodule formation is under tight regulation.

Ammonium, besides being the final product of the nitrogen fixation, is one of the main regulators of nodulation, having a positive or negative impact upon the various stages of symbiosis (Patriarca et al. 2002; Barbulova et al. 2007). At low concentrations, the combined nitrogen that is bioavailable in the form of ammonium, nitrate or urea may have a positive effect upon formation and development of nodules. At high concentrations, the excess of nitrogen sources in the soil reduces the nodulation, and in particular, the number of infections in roots (Zahran 1999; Barbulova et al. 2007). As such, ammonium is one of the primary regulators of the symbiosis process.

Nitrogen, in the form of NH_4NO_3, was found to be a powerful inhibitor of the curling of root hairs, of cortical cell division and the infection thread formation. In later stages of nodule development, adding ammonium inhibits even the activity of the nitrogenase enzyme complex, through influence of the leghemoglobin activity.

In the case of indeterminate nodules, the endogenous ammonium produced through N fixation is a positive regulatory signal which is necessary for a proper development; in fact, this type of nodules, when induced from a strain of *S.meliloti* mutated for the *nif* gene for nitrogenase (therefore, unable to fix nitrogen), starts the initial process of organogenesis which remains incomplete, ending with a precocious senescence (Hirsch and Smith 1987). This is not replicated in the case of determinate nodules, which do not show developmental defects even when infected with mutants of the nitrogenase *nifH*; this excludes the ammonium resulting from the symbiosoma for a signaling role in the regulation of organogenesis in determinate nodules (Patriarca et al. 2002).

Ammonium also affects the signal that regulates the number of nodules; plants roots infected with a Fix- strain, unable to fix nitrogen, make a greater number of nodules compared with the number that normally arises in plants infected with a *Fix+* strain (Patriarca et al. 2002). Legumes not only possess tightly-regulated molecular machinery devoted to organogenesis of nodules, but also have the means to control the efficiency of the process and the particular number of nodules formed. This type of auto-regulation

system is based upon systemic signals and *feedback* mechanisms correlated with the final output of symbiosis that is ammonium production.

Auto-regulation is a mechanism that controls the number of nodules that are formed in the plant roots even earlier, soon after the infection. This process regulates the density of nodules and their disposition across the root. This process comes across through molecular signaling, codified from the aerial plant of the plant a few hours after infection by the bacteria, allowing only the primary steps of infection, assuring the completion of the nodule organogenesis and aborting any later infection events. In *Lotus japonicus*, the gene isolated as responsible for auto-regulation is *HAR1* (Hypernodulation aberrant root formation), which codifies for a kinase-like protein rich in leucine repeats, with a single transmembrane domain and a serine/threonine phosphorylation domain (Krusell et al. 2002). Orthologues of HAR1 have been found in other legumes, like *GmNARK* (glycine max Nodule Autoregulation Receptor Kinase) in soybean*, PsSym29* (*Pisum sativum* Symbiosis 29) in Pisum and *MtSUNN* (*Medicago truncatula* Super Numeric Nodules) in Medicago. Mutations in these genes result in hyperformation of nodules and are able to nodulate even in the presence of high concentrations of nitrate. The orthologue of these genes in *A. thaliana* has been identified as *CLAVATA1* (CLV1), a gene that negatively regulates the formation of the apical and floral meristem. In particular, CLV1 is involved in the shoot meristems in the maintenance of the tight balance between un-differentiated stem cells and differentiated cells that is a requisite for a correct shoot developmental plan (Clark et al. 1997). CLV1, a leucine-rich receptor kinase signaling protein, is involved in a cell to cell communication by binding the short CLE peptide CLV3 and limiting its diffusion at the shoot meristematic center (Fig. 8). By analogy, a similar regulatory system between root and shoot has been proposed for the autoregulation-responsible process in legumes. In the current model root signals represented by short CLE peptides, induced by rhizobium infection or nitrate provision, are transported to the shoot where interact with HAR1 determining the regulation of nodule number (Okamoto et al. 2009)

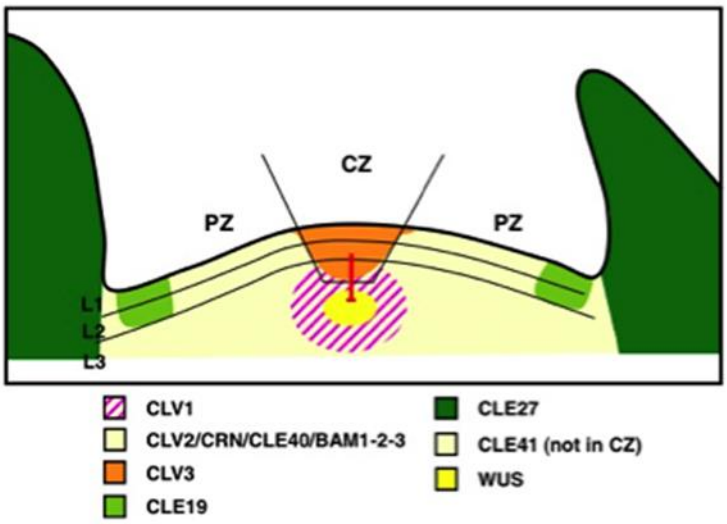


Fig. 8: Schematic representation of the shoot apical meristem (SAM) expressed *CLE* genes and genes involved in the CLAVATA pathway in the SAM. The central zone (CZ) harbors the stem cells that are specified by *CLV3* expression and the organizing center is marked by *WUS* expression and surrounded by *CLV1* expression. Next to the CZ are the peripheral zones (PZ) where organ primordial are initiated.

In general, it is possible to classify three categories of hypernodulating mutants: the nitrogen-tolerant mutants (nts), which make an elevated number of nodules across the entire length of the root; the ethylene-insensitive mutants, which make nodules that grow near each other in a delimited zone of the root; and the light-insensitive mutants, which produce twice the number of nodules of wild-type plants, but preserve the same disposition and location alongside the roots. Other actors implicated in the auto-regulation process are phytohormones, brassinosteroids, abscisic acid and jasmonate, which seem to act down-stream of the signaling system (Oka-Kira and Kawaguchi 2006).

1.2.5 Role of Hormones in nodule organogenesis

Nodule development requires multiple signals from both plant and rhizobia, but at a very elemental level mirror many processes of common plant development: many cells divide and differentiate, there is development of vascular tissue and responses and regulation to external factors like stress and nitrate. As such, it is a safe assumption that the role of multiple phytohormones in nodulation regulation is due to pleiotropic responses, as they regulate plant development processes per se and do not have a direct effect upon nodule organogenesis.

Abscisic acid (ABA) is a naturally occurring compound in plants, derived from the mevalonic acid biosynthesis pathway that also leads to plant sterols, brassinosteroids and gibberellins. It plays crucial roles in various aspects of plant growth and development, including the mediation of responses to environmental stress such as cold, drought and high salinity. In nodulation, ABA has been reported to play multiple negative roles in different stages of nodule development. In *Pisum sativum*, ABA application through root irrigation was shown to inhibit nodule numbers; ABA applied to *wild-type* and supernodulating NOD1-3 soybean reduced both the number of nodules and isoflavonoid levels, which are responsible for the activation of the transcription of nodulation factors and triggering nodule organogenesis. Later reports reveal that ABA inhibits all phases of nodulation from nodule initiation, development and function, but seem to do so independently of nodulation control status.

The effect of ABA upon the nodulation of plants that form indeterminate nodules (white clover) and determinate nodules (*Lotus japonicus*) has been reported; in both legumes and types of nodules, root application of ABA reduces the number of nodules. Since root hair deformation was observed, ABA is believed to block the steps down-stream of root hair deformation. Consistently, application of abamine, an ABA biosynthesis inhibitor, increases the number of nodules, making ABA likely to be involved in the auto-regulation of nodule numbers (Suzuki et al. 2004).

ABA also affects nodule development; treatment with this phytohormone results in arrested, small and infective brownish nodules with early degeneration of bacteroid tissue. The nitrogenase activity of nodules treated with ABA was lower compared to wild type in *Faba vulgaris*, pea and *Lotus japonicus*; this led to the hypothesis that ABA stimulated an abrupt stress situation mimicking severe drought, which led to leghemoglobin reduction, accumulation of oxygen and inactivation of the nitrogenase complex (Gonzalez et al. 2001; Tominaga et al. 2010). In addition to that, ABA might create an oxygen barrier in nodules that resulted in the observed decline of nitrogen fixation. Histological sections of nodules inoculated with rhizobia expressing green fluorescence protein (GFP) revealed a fluorescent green “shell-like” structure on the outer layer of spherical *Lotus japonicus* nodules treated with ABA (Fig. 9). Bacteroids in the nodule interior failed to express GFP, suggesting that the possible degeneration of bacteroid tissues may be caused by an ABA-induced oxygen barrier

(Biswas et al. 2009). This may relate to systemic stress responses in legume nitrogen control, where stress treatment of one separated root portion results in a systemically transmitted drop of nitrogen fixation ability in a second root portion. Interestingly, nodulation is not suppressed in that situation, suggesting that ABA mechanisms of nodulation control are local, while some act systemically. Of course, general plant fitness effects would presumably be systemic.

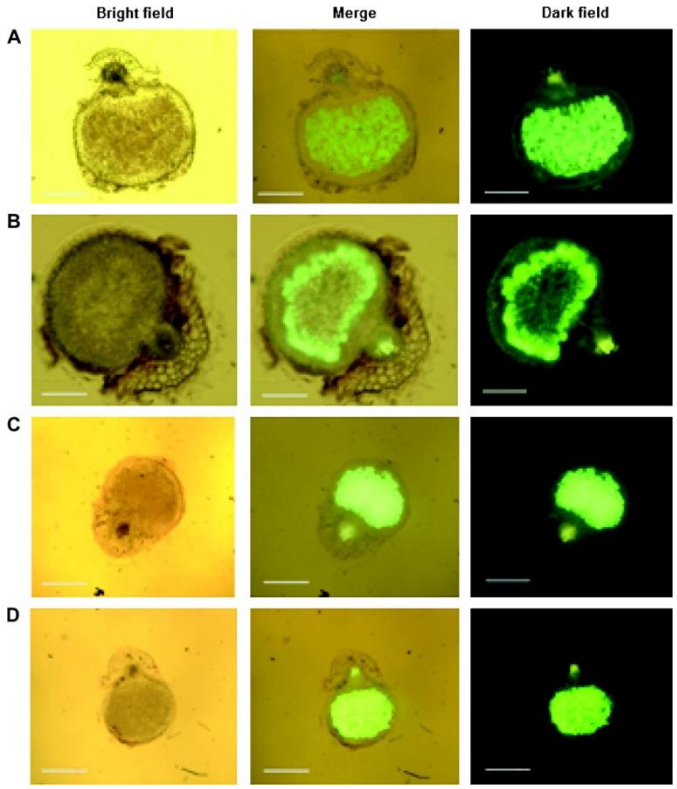


Fig.9: Histology of Nodules Viewed under UV Light Microscope (100× Magnification).(A) MG-20 nodules of plants grown at 0 µM ABA.(B) MG-20 nodules of plants grown at 50 µM ABA.(C) Beyma nodules of plants grown at 0 µM ABA.(D) Beyma nodules of plants grown at 50 µM ABA. Scale bar = 50 µm. Increased ABA concentration and accumulation in the nodule does not seem to impair nodulation. (Biswas et al. 2009)

The possible role of ABA in auto-regulation of nodulation was further investigated in a supernodulating soybean mutant *nts382*, which has an ever strong supernodulation phenotype than the NODI-3 mutant. The basal levels of ABA in *nts382* are lower than the wild type plants, and increased even further in wild type plants roots following inoculation with *Bradyrhizobium*. The concentration of ABA in the shoot increased at the onset of autoregulation in the wild type, while the same was not verified in *nts382*; similarly, the root ABA-to-cytokinin ratio was found to be consistently higher in the wild type compared to

nts382 (Caba et al. 2000). This ratio has been suggested to be involved in the root-to-shoot signaling and symbiotic photosynthetic gas exchange in alfalfa (Goicoechea et al, 1997). A model was proposed to explain the possible influence of ABA-to-cytokinin ration in autoregulation of nodulation. In this model, inoculation-induced changes in the xylem resulted in a decreased ABA-to-cytokinin ratio that triggers the synthesis of ABA when moved up to the leaf. This ABA was speculated to be translocated to the root to autoregulate nodule development. However, it was later found that ABA may not be directly involved in autoregulation of nodulation; an ABA insensitive mutant of *Lotus japonicus* did not have altered autoregulation of nodulation, and the applied effect of nodulation was local in a split root experiment with *Lotus japonicus* (Biswas et al. 2009).

An ABA insensitive mutant in *Lotus japonicus*, *Beyma*, was isolated based on the growth of root length. *Beyma* is an ABA response mutant that displays insensitivity to the inhibition of germination, stomata closure and nodulation. *Beyma* forms a number of nodules similar to the wild type – indicating autoregulation of nodulation was not affected. However, nodules formed in *Beyma* were smaller than wild type and had reduced ability to fix nitrogen. This led to the proposal that ABA is not directly involved in autoregulation, but has a role in nodule growth rather than the control of the early signals triggering nodulation (Biswas et al. 2009).

Auxins were the first class of major plant hormones discovered to be central to regulation of plant growth and development at all levels. The most important member of the auxin family is indole-3-acetic acid (IAA), a native auxin in plants, derived from the phenyl-propanoid biosynthetic pathway. The highest auxin concentration was found in cells undergoing cell division, elongation, differentiation and vascular bundle formation. Therefore, auxin has been speculated to play a significant role in nodule development (Pasternak et al. 2002). Auxins are produced mainly in the shoot and moves to the root by an active transport process involving efflux protein complexes. These proteins may regulate the auxin concentration in the plant. These are not evenly distributed along cell membrane and are subject to dynamic reallocation. Compounds like NPA (1-N-aphtylphthalamic acid) and TIBA (tri-indobenzoic acid) inhibit the acropetal auxin transport. Rhizobium-derived nod factors and several classes of flavonoids have been reported to have a similar effect on auxin transport (Mattsson et al. 1999).

Early experiments suggested that the ratio of auxin-to-cytokinin in the root was responsible for the initiation of cortical cell divisions and nodule formation. This ratio was lowered in soybean hypernodulating mutant compared to wild type, indicating that hormones balance is important for regulating nodule number. Various plant compounds like ethylene, cytokinin and peroxidase could inhibit auxin transport, which could lead to local shifts in the plant's auxin-to-cytokinin ration (Caba et al. 2000). The use of an auxin-regulated soybean promoter has given new insights into auxin distribution and expression in legumes (Li et al, 1999). The GH3 promoter is active in tissue with high level of auxin, and has quickly and seemingly presented specific responses to various auxin concentrations. Its expression, monitored by GUS staining, was detected in dividing cells of the nodule and lateral roots. At early stages of nodulation, inferred auxin levels increased in early dividing cortical cells and decreased in differentiating nodule primordial and vascular tissue (Pacios-Bras et al. 2003). Similar observations were made in *Medicago truncatula* AUX1-like genes (MtLAX). The *MtLAX* genes are expressed in nodule primordial at early stages of nodule development and vasculature emerged at the later stage (de Billy et al. 2001).

The role of auxin in nodulation is closely related to the development of other root structures like lateral roots, as these structures undergo a similar development program which involved cell division and differentiation. Both lateral roots and nodules are regulated by auxin-to-cytokinin ratio, but in an opposite way. An increased in auxin stimulated lateral formation while an increase of cytokinin concentration or inhibiting auxin transport induced the development of pseudo-nodules. The supernodulating mutant *astray* in *Lotus japonicus* has a normal frequency of lateral roots, suggesting that the pathway regulating nodule and lateral root formation may share a common evolutionary origin, but with the existence of nodule-specific regulators (Nishimura et al. 2002).

Cytokinins are a class of plant hormones active in promoting cell division, and are also involved in regulation of many physiological processes during plant development, growth and adaptation to environment conditions. Cytokinins are implicated in the control of root architecture development, including root nodulation. One of the earliest indications was the observation of pseudo-nodule structures in legumes (pea and alfalfa) after application of exogenous cytokinins; this was verified even in non-legumes like tobacco (Hirsch and Fang 1994). These physiological studies revealed a role for this hormone for the control of root

architecture and nodule development. Exogenous application of cytokinins on legume root induced responses similar to Nod factors (Bauer et al, 1996). This includes cortical cell divisions, amyloplast deposition and induction of early nodulin gene expression. Transfer of trans-zeatin secretion gene (tzs) into Nod- bacteria and non-symbiont bacteria was sufficient to induce nodule-like structure formation at low frequency in alfalfa (Cooper and Long 1994). Similar observation can be seen when cytokinin metabolism was altered by transgenic overexpression of cytokinin oxidase (Lorteau et al. 2001). On the other hand, suppression of a cytokinin receptor by RNA interference reduced nodulation in *Medicago truncatula* (Eckardt 2006).

Further evidence that cytokinins are crucial for nodule growth comes from the work with *Lotus japonicus*. A gain-of-function mutant allele of a *Lotus japonicus* gene for histidine kinase (*Lhk1*) that leads to spontaneous nodule formation in absence of rhizobia and a loss-of-function allele, *HYPERINFECTED1 (HIT1)* of the same *Lhk1* gene were identified. *HIT1* mutant failed to establish nodules and lead to a hyper-infected phenotype of roots (Murray et al. 2007). This demonstrates unequivocally that cytokinin signaling is necessary and sufficient to induce cortical cell divisions and nodule organogenesis. Studies also indicate that cytokinin has a role in the differentiation of lateral roots in legumes. In non-nodulating plants such as Arabidopsis, cytokinins have been shown to negatively regulate the initiation and growth of lateral roots through the partially redundant function of cytokinin receptors AKH2 and AkH3 (Nishimura et al. 2004). In legumes, reducing cytokinin content and/or blocking *MtCRE1*-signalling also results in increased lateral root formation under non-symbiotic conditions. (Lohar et al. 2004; Gonzalez-Rizzo et al. 2006). Furthermore, promoter-GUS fusions activity studies show that transcription of these genes localizes to lateral root promordia of MtCRE1. Collectively, this data points to cytokinin as a central signal controlling lateral organ differentiation in the root and suggests that a local increase in cytokinin status – wheter achieved by a change in cytokinin metabolism and/or perception – induces organogenesis while repressing lateral root formation.

Ethylene promotes root hair elongation in roots and interferes with the fate of root epidermal cells, with high concentrations of ethylene committing Arabidopsis atrichoblast to form root hairs. Etyhlene also promotes auxin biosynthesis in roots, altering the auxin root tip gradient. This results in increased cell division and inhibition of cytokinin-dependent cell

elongation/differentiation (Tanimoto et al. 1995; Cao et al. 1999; Ortega-Martinez et al. 2007). In legumes, ethylene appears to be mostly produced in the roots and seems to be significantly induced by light; simultaneously, the number of nodules decreases, suggesting a role of ethylene as a repressor of nodulation (Lee and Larue 1992; Lee and Larue 1992). Treatment of pea plants with exogenous ethylene represses nodule organogenesis as infection threads were arrested in the inner cortex (Lee and Larue 1992). This arrest could reflect a role for ethylene in defining the radial epidermiscortex boundary in the susceptible zone. Ethylene inhibition would need to occur very early in the symbiosis-signaling pathway, since calcium spiking in response to treatment with Nod factor is inhibited by addition of ethylene precursor (Ehrhardt et al. 1996). Further evidence for ethylene's negative role upon nodulation has emerged from rhizobial studies. Many rhizobacteria encode ACC deaminase (accD) which catalyses degradation of the ethylene precursor 1-aminocyclopropanme-1-carboxylic acid (ACC), tuning down the plants defense response. Consistent with this function, *accD* mutants induce fewer nodules compared to the wild-type strain. Furthermore, increased expression of accD is observed during differentiation of *Mesorhizobium loti*, suggesting a function during symbiosis (Ma et al. 2003; Uchiumi et al. 2004; Glick 2005).

Ethylene also influences the positioning of nodule primordia. Nodule primordia are precisely located in a position opposite to protoxylem poles on the root, and their number is tightly controlled. The ethylene-forming enzyme ACC oxidase transcripts are more abundant in cells facing protophloem poles, positions where nodules are unlikely to develop (Heidstra et al. 1997; Ding and Oldroyd 2009). Additional ethylene effects on nodule meristems and infection were inferred from semiaquatic legumes such as *Sesbania rostrata*, which can develop both determinate nodules by crack entry and indeterminate root nodules by root hair/infection thread infection, depending on the ethylene concentration. Together with gibberellin and oxygen peroxide, ethylene contributes to cell death and creation of infection pockets in the root cortex characteristic for crack entry (Lievens et al. 2005). This intercellular type of infection mechanism has also been observed in Lotus, and studies of the nena mutant infected by crack entry suggest that ethylene promotes crack entry in Lotus (Karas et al. 2005; Groth et al. 2010; Madsen et al. 2011). Genetic support for the role of ethylene in legumes symbiosis is relatively scarce; a genetic screen for components of the

nodulation pathway identified a Medicago mutant line (*skl*) with about 10-fold increased nodule number (Oldroyd et al. 2001; Penmetsa et al. 2008). A careful analysis of its hypernodulation phenotype indicated that *skl* is insensitive to ethylene (Oldroyd et al. 2001; Penmetsa et al. 2008). Further characterization of *skl* alleles uncovered mutation in a homolog of Arabidopsis ethylene-insensitive (EIN2), an essential component of ethylene signaling, confirming ethylene's role as a repressor of nodulation, or alternatively, that a decreased in EIN2 activity is required for nodulation. In-parallel transgenic approaches conducted in Lotus provided further evidence for ethylene as a negative regulator of nodulation. Transgenic Lotus plants constitutively overexpressing a mutate version of the melon ERS1 ethylene receptor or the Arabidopsis etr1-1 that cannot bind ethylene have reduced ethylene sensitivity. These transgenic lines had increased numbers of infection threads and increased numbers of primordial which were often mislocated (Nukui et al. 2004; Lohar et al. 2009).

Ethylene's impact upon nodulation is not that straightforward: a genetic screen conducted in soybean identified several lines showing strong ethylene insensitivity surprisingly, these lines showed reduced number of nodules compared to wild-type. A similar genetic screen in Lotus identified the enigma mutants showing a severe ethylene insensitivity phenotype. These enigmatic mutants appear to be defective in a Lotus homolog of Arabidopsis EIN2, and unlike Medicago *skl* mutants, these mutants also display a significant reduction in nodule number (Schmidt et al. 1999; Gresshoff et al. 2009). Through comparison of the Lotus genome resources, a set of genes corresponding those in Arabidopsis involved in ethylene biosynthesis, signaling and regulation were identified (Fig. 10). A quick overview suggests that the Lotus and Arabidopsis ethylene pathways are comparable, although not necessarily identical. The differentially regulated gene family of ACC synthases (ACS), which mediates the penultimate rate-limiting step of ethylene synthesis in Arabidopsis and Lotus, has three Lotus subtypes. So far, the subtype 1 ACS1 and ACS2 have not been found in Lotus, while ACS6 is present in two copies. In Arabidopsis, ACS6 is likely to be active during hypoxia (Peng et al. 2005), while ACS1 is a nonfunctional unit that regulates ACS activity (Tsuchisaka et al. 2009); these differences may be due to a unique regulation of ACS activity in Lotus, due to the context of nodulation.

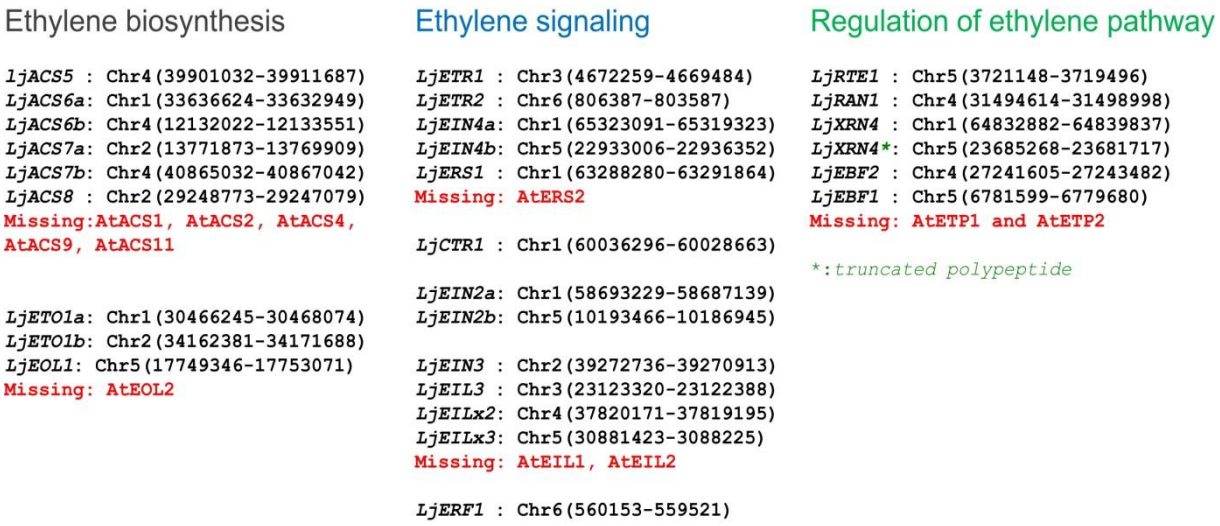


Fig. 10: Comparison of *Lotus* and *Arabidopsis* genes regulating ethylene pathways. Lotus has 6 of the 11 ACS genes involved in ethylene biosynthesis, as well as 5 homologues for the 7 Arabidopsis genes involved in regulation including a truncated version of *XRN4*. Arabidopsis and Lotus show important differences in signaling, encoding the same numbers of ethylene receptors, but there is no homologue for *ERS2* and *EIN4* is present in two copies (Desbrosses and Stougaard 2011).

The Arabidopsis subtype 2 ACS5 plays a key role during cytokinin-induced ethylene emission, and cytokinin also promotes ethylene biosynthesis in pea, suggesting a similar role for Lotus ACS5 (Vogel et al. 1998; Lorteau et al. 2001). Finally, the two ACS7 gene products, representing subtype 3, may, like Arabidospsis ACS7m interact with XBAT32, an E3 ubiquitin ligase, and affect lateral root development through a mechanism involving ethylene biosynthesis (Prasad et al. 2010). This level of post-translational regulation may be conserved in Lotus.

Homologues of both Arabidopsis ETO1 and EOL were found in Lotus, suggesting that ETO1/EOL-mediated post-translational mechanisms regarding ethylene biosynthesis may also be conserved in Lotus. The Arabidopsis ETO1/EOL proteins interact with the C-terminal domain of subtype 2 ACS and with cullin 3A through their BTBT (Broad complex/Tramtrack/Brick-a-Brack) and a TPR (tetratricopeptide) domain. The resulting protein complex interacts with ubiquitin ligase and targets subtype 2 ACS for degradation (Christians et al. 2009).

In Lotus, there are two copies of EIN2; in comparison there a single copy *Medicago* and at least three copies in soybean. In Arabidopsis, EIN2 is an essential component of the ethylene-signaling pathway, as a loss-of-function EIN2 mutation leads to complete

insensitivity to ethylene (Guzman and Ecker 1990). If EIN2 has a specific function in termination of the determinate nodule meristem, and deregulated repression of additional EIN2 copies might lead to fewer nodules and explain the opposing phenotypes of enigma (Gresshoff et al. 2009) and *Medicago skl* mutants (Penmetsa et al. 2008). The genes that code the two F-box proteins ETP1 and ETP2 that control the abundance of EIN2 in *Arabidopsis* are either missing of too divergent to be detected by bioinformatics in legumes (Qiao et al. 2009); EIN2 mays thus be regulated in a different way in legumes. There are also differences for the transcriptional regulators EIN3 and EIN3-like coding genes, which seems to suggest additional functions compared to the Arabidopsis regulators (Binder et al. 2007). One of the targets of EIN3 is the regulation of AP2-type transcription factor ERF1, which in turn regulates the expression of ethylene downstream responsive genes (Solano et al. 1998). Homologous of these AP2-type family genes might regulate the ethylene-signaling pathway in Lotus and differences in the expression of these genes may reflect the role of ethylene at different stages of nodule development.

The plethora of actions from this and other hormones upon the early stages of nodulation is reviewed below (Fig. 11)

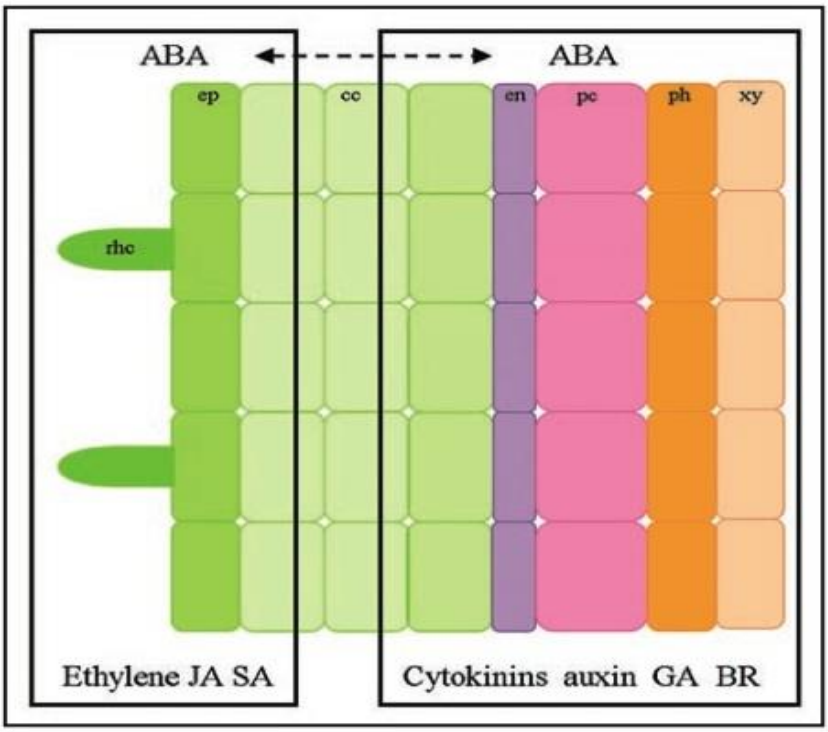


Fig. 11: In the epidermis, defense hormones such as ethylene, JA and SA as well as the stress hormone ABA negatively regulate Nod factor induced calcium spiking, early nodulin gene expression and infection thread initiation. In the cortex and in the pericycle cells, the balance of cytokinins, ABA, and auxin dictate whether lateral roots or nodules will be initiated. GA and BR are also involved in the formation of the nodule primordial. ABA has dual roles in regulating epidermal and cortical nodulation processes and as such may facilitate the coordination of the epidermal and cortical nodulation processes and as such may facilitate the coordination of the epidermal and cortical programs. Rhc, root hair cell; ph, phloem; xl, xylem; JA, jasmonic acid; SA, salycilic acid; ABA, abscisic acid; GA, gibberellic acid; BP, brassinosteroid.

1.2.6 Role of Nitrate as an early Regulator of Nodulation

The condition of N starvation is a pre-requisite for the occurring of the nodulation process as the presence of sufficient amount of N sources in the growth media (nitrate, ammonium, urea etc) strongly inhibits the nodule organogenesis program. This is simply due to the fact that legumes prefer to take up and assimilate N source readily available in the soil rather than starting the very expensive process of nodule organogenesis and N fixation. In certain genotypes *of Medicago sativa*, nitrogen starvation alone is sufficient to induce the

development of empty (bacterium-free) nodules (Truchet et al. 1989). Nitrogen starvation is also a mandatory condition for nodule development in spontaneous nodulation mutants recently isolated in *L. japonicus* (Tirichine et al. 2006). Among the N sources the effects played by nitrate on the nodulation process have been already reported in the beginning of 1990 (Carroll and Mathews 1990; Caetano-Anolles and Gresshoff 1991).

The nitrate peculiarity is based on the fact that it may behave not only as a nutrient, but also as a signal, being able to trigger signaling pathways controlling lateral root development at both systemic and local level, respectively. (Zhang and Forde 1998).

Nitrate effect upon nodulation, shares many parameters with nitrate's control upon secondary root development, its control being exerted both at a local and systemic level. (Fujikake et al. 2003; Omrane and Chiurazzi 2009; Jeudy et al. 2010). Low nitrate concentration in the growth medium might enhance nodule formation, whereas high concentrations (>1 mM NO_3^-) have an inhibitory effect upon nodule formation. Genetic evidences obtained with plant *nar* mutants indicate that these effects don't depend on nitrate assimilation by nitrate reductase; therefore, this inhibitory effect is unlikely to be due to a nutritional effect (Carroll and Mathews 1990). The high KNO_3 concentration effect is associated with two clear-cut early phenotypes reported in *M.loti* infected *Lotus japonicus* plants: inhibition of cortical cell division and down-regulation of the NIN gene expression through a HAR1 dependent pathway (Barbulova et al. 2007).

While the overall impact of nitrate upon nodule formation has been reported, the mechanisms and factors involved in the multiple signaling pathways leading to nitrate-dependent nodule organogenesis inhibition are still largely unknown. Interplay between nitrate and auxin has been reported for both secondary root and nodule organogenesis processes. In the case of secondary root elongation and early development; the effect of local nitrate supply was not observed in Arabidopsis *axr4*, an auxin-resistant mutant, suggesting an overlap between the nitrate and auxin response pathways. More recently a direct involvement of the nitrate transporter NRT1.1 in the auxin distribution controlling the secondary root elongation, linked to the external nitrate concentration sensing, independent from nutritional status has been reported in *A. thaliana* (Krouk et al. 2010; see below). The high-affinity nitrate transport complex NRT2.1-NAR2.1 also participates in regulating lateral root development, in a process independent of its uptake functions and the nitrate

availability (Little et al. 2005). This signal role of nitrate has also been reported in legumes, as the *Medicago truncala* MtNPF1.7/NIP-LATD, recently characterized as a high-affinity nitrate transporter, had been previously reported as involved in lateral root and nodule development and primary root meristem maintenance (Bright et al. 2005; Bagchi et al. 2012).

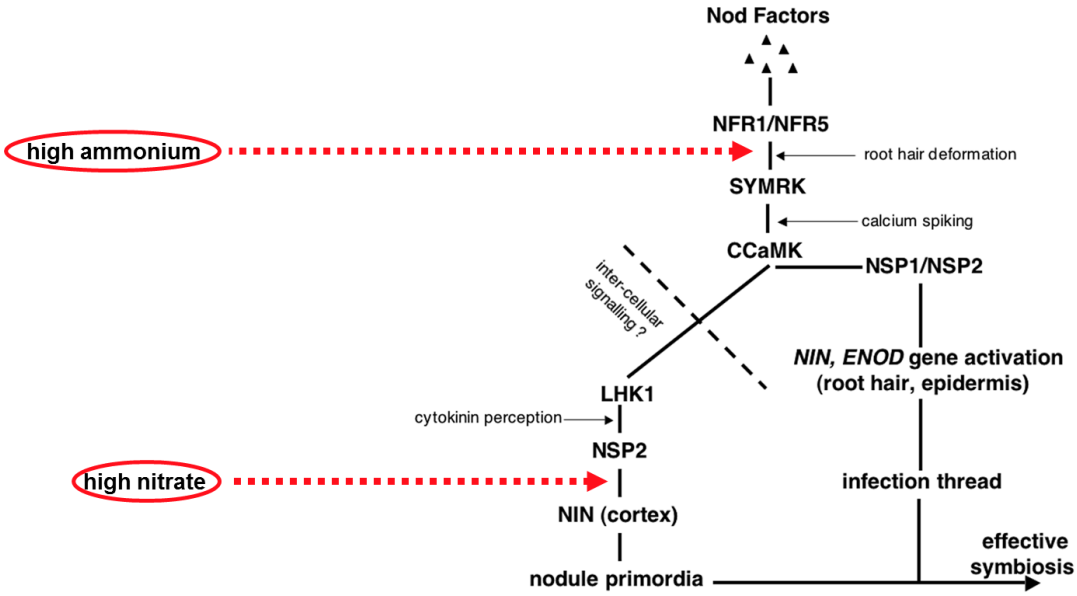


Fig. 12. Nitrogen sources and nodule formation signaling pathways. Nitrate regulates NIN expression and arrests nodulation after root hair formation and before cortical cell division; ammonium on the other hand arrests nodule formation as early as root hair deformation (Barbulova et al. 2007).

An auxin-burst-control (ABC) hypothesis (Gresshoff, 1993) was previously presented to explain nitrate inhibition and autoregulation of nodule numbers; that hypothesis suggested that Nod-factor perception alters axial and radial auxin transport, allowing the initiation of cortical cell divisions through a shift in the local auxin-to-cytokinin ratio. Such changes alter pericycle and epidermic responses, leading to nodule formation. Once an increased amount of nodule initiation has occurred, the shoot responds through an increase in translocation of auxins, leading to an auxin burst, which in turns is inhibitory for further nodule initiation. The ABC hypothesis predicts that nitrate increases the auxin sensitivity of root cortical cells and thus in the presence of nitrate, cortical cells are strongly prevented from sensing the Nod-factor-related auxin decrease. Experimental data reported that a 45% increase of IAA content was actually observed in inoculated soybean plants frown in 1mM

nitrate, while no apparent increase in root auxin was observed in the presence of high nitrate concentrations (Aung et al. 2006; Omrane and Chiurazzi 2009).

It is well reported that the nod genes in *Rhizobium* are activated by signal compounds – flavonoids and isoflavonoids. Soybean hypernodulating mutants demonstrated increased concentration of flavonoids; the concentration of flavonoids was decreased after application of nitrate to the roots, an effect that was both observable at local and systemic level – implicating that nitrate might regulate organogenesis by decreasing flavonoid signaling by the host plant (Cho and Harper 1991). An inoculation-stimulated ethylene release pathways has been associated with nodule regulation, with reduction of ethylene synthesis increasing nodulation, and while nitrate has been reported to induce ethylene, treatement with nitrate also influences concentration of auxin, cytokinin and abscicic acid. Since these phytohormones may be secondary regulators of nodulation, nitrate signalling might activate through various different pathways (Caba et al. 2000).

Hypothesis like the aforementioned ABC hypothesis is one of the models that have been proposed to explain systemic long-distance nitrate-dependent signaling, involving a shoot-derived auxin signal. Still, the individual mechanisms and actors remain unknown.

A new role through which nitrate regulates nodule formation has been recently proposed: nitrate-responsive CLE peptides may be the main actors in transduction of the nitrate root signal to the shoot-dependent mechanisms that governs auto-regulation of nodule numbers in *Lotus japonicu*s, soybean and *Medicago truncatula*, thus regulating nodulation (Okamoto et al. 2009; Mortier et al. 2010; Reid et al. 2011)

The mechanism of auto-regulation of nodulation, which, in normal conditions regulates the number of nodules formed after inoculation with rhizobia, is based on a system of long distance signaling according to the current model. A signal that starts in the plant root after the inoculation is transmitted to the shoot, where it triggers an HAR1-controlled signal, which starts from the shoot and inhibits systemically the nodulation. The level of homology between *HAR1* and the Arabidopsis *CLAVATA1* suggests that the two might share a similar mechanism of action; *CLAVATA1* controls the equilibrium between differentiated and pluripotent cells at the level of the shoot apical meristem through recognition of certain signaling peptides (CLE) through the kinase domain of *CLAVATA1*. CLE short peptides represent a large ligand family isolated from plants involved in a broad range

of developmental processes (Wang and Fiers 2010). A systematic analysis of the expression profiles of 39 LjCLE genes identified in the *L. japonicus* genome (Sato et al. 2008) has been reported (Okamoto et al. 2009). This allowed the identification of two LjCLE genes significantly early up-regulated in inoculated roots and two of these, LjCLE-RS1 and LjCLE-RS2 (RS = Root Signal), when overexpressed in *L. japonicus* transgenic roots significantly inhibited the nodulation (Okamoto et al. 2009) in a HAR1-dependent manner. Interestingly, LjCLE-RS2, expressed specifically in the root tissue is also induced in non-infected plant roots when nitrate is added to the growth media with an increasing level of expression in increasing nitrate concentration conditions. These results and the identification of this CLE root signal, that respond to both biotic and abiotic stimuli, allowing the assembly of a hypothetical model in which the phenotypes of auto-regulation and tolerance to nitrate are tied to a molecular mechanism which involves at least the factors HAR1 and CLE-RS2 (Fig. 13).

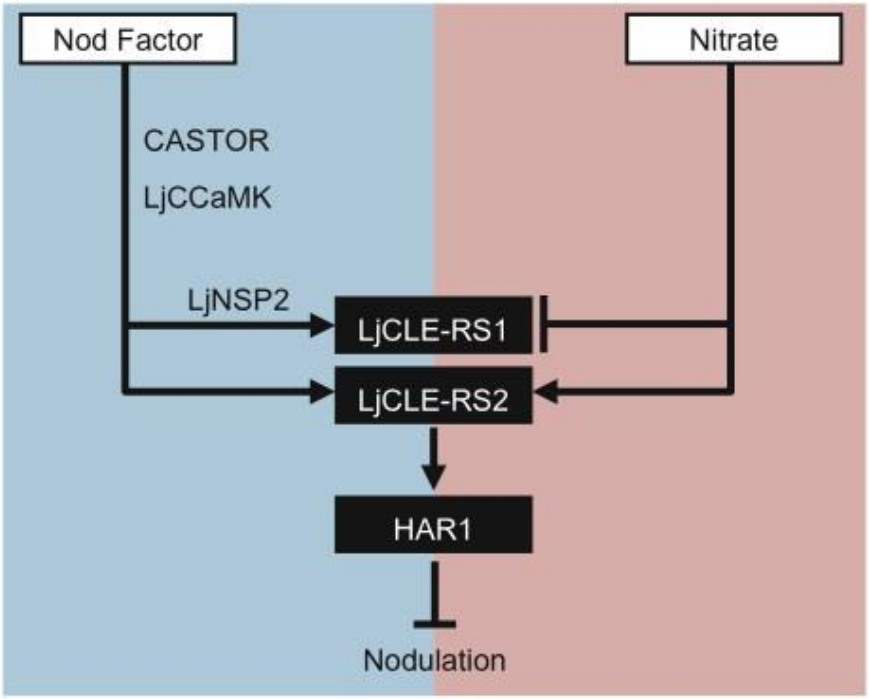


Fig. 13: HAR1/CLE-RS2 auto-regulation model. LjCLER-RS1 and LjCLE-RS2 are up-regulated by nodulation, LjCLE-RS1 being responsible for transmission of downstream signaling while LjCLE-RS2 is recognized by HAR1, triggering an auto-regulation response that inhibits further nodule formation. High nitrate concentrations also increase LjCLE-RS2 expression, triggering HAR1 and auto-regulation pathways, impairing nodulation (Okamoto et al. 2009).

Another early effect of high external Nitrogen availability on the nodule formation program has been reported by Omrane et al. (2009). In this case was the competence for nodulation of *L. japonicus* plants pre-incubated on low (10 µM NH_4NO_3) or high (10 mM NH_4NO_3) N conditions prior to the M. loti inoculation to be evaluated. The results indicated that the different treatments could affect the predisposition of the plants for starting the nodule organogenesis program once transferred on low N permissive conditions. In particular, the plants pre-incubated for 10 days on 10 mM NH_4NO_3 conditions showed a nodulation capacity that was about 50% of that of plants pre-incubated on 10 µM NH_4NO_3. Interestingly, the inhibitory effect was observed even in plants that, after the 10 days high N pre-incubation, were transferred on low N for 6 days before performing the M. loti inoculation, indicating that this negative effect on the nodulation competence was due to a long term action. This suggested that the inhibitory effect was correlated to a systemic change of the general N nutritional state of the plants that was transduced through a signaling pathway to the root nodulation machinery. Moreover, the phenotypical observations were coupled to a transcriptomic analysis leading to the identification of a number of genes candidates for such an action of link between nutritional N status and nodulation capacity (Omrane et al. 2009)

Several intricate regulatory networks have been reported in plants for integrating N assimilation and developmental pathways such as root branching, leaf growth and flowering time (Marschner 1995 ; Scheible et al. 1997 ; Zhang and Forde 1998 ; Stitt and Krapp 1999). Thus the response to the presence of N results in important changes at the growth/phenotypic level. Another Affymetrix geneChip analysis performed in *M. truncatula*, confirmed a strong systemic influence of the N supply on the profile of gene expression in nodulated roots (Ruffel et al. 2008). In particular, a severe down-regulation of 200 nodule-related transcripts was observed, in a split-root system, when one side of the roots was treated with 10 mM NH_4NO_3 (Ruffel et al. 2008)

1.2.7 Role of Nitrate as late Regulator of Nodule functioning

Nitrate plays also an important role as regulator of legume nodules activity as it is known that a few days after nitrate exposure, nodule activity is almost completely lost (Schuller et al. 1988) and the nodules become senescent (Matamoros et al. 1999), but the

mechanisms through which this action takes place is still controversial. The nitrate ion itself, nitrite, or nitric oxide (NO) have implicated by either blocking leghemoglobin (Kanayama and Yamamoto 1990; Kato et al. 2010) or by triggering a regulatory network at the gene expression level (Neill et al. 2008; Wilson et al. 2008; Meilhoc et al. 2011). NO production is stimulated in nodules by nitrate and nitrite and inhibited by a nitrate reductase inhibitor (Horchani et al. 2011). Both plant and bacteria nitrate reductase and electron transfer chains are involved in NO synthesis and a nitrate-NO respiration process in nodules could play a role in the maintenance of the energy status required for nitrogen fixation under oxygen-limiting conditions.

A further hypothesis is that the effect of nitrate is mediated by a closure of the oxygen diffusion barrier or a reduction in oxygen permeability facilitated by unspecified mechanisms (Vessy and Waterer 1992; Minchin 1997). However, no clear time course study has been carried out showing that a decrease in nodule O_2 uptake precedes and induces decline in nitrogenase activity. Of course, the decreased respiration and lowered O_2 influx into the nodule after nitrate provision might also be the result of a decline in nitrogenase activity induced by a different mechanism.

Another reason for reduction of nitrogenase activity under nitrate impact might be related to a local or systemic assimilate diversion (Fujikake et al. 2003). The growth of emerging nodules immediately and completely stopped after nitrate provision in soybean. Carbon allocation within the plant was profoundly influenced with less going to the nodules. However, there is no unequivocal proof that this is the primary factor inducing the decline in nodule activity.

In addition, there may be a shoot-mediated mechanism of down-regulating nitrogen fixation dependent on a phloem mobile signal reflecting the nitrogen satiety status of the leaves (Naudin et al. 2011). Such a signal might contain nitrogen as nitrogen travels quickly from leaves to nodules (less than 15 min) (Fischinger et al. 2006).

However, a very recent report indicates that the nitrate induced decline in nodule activity is mirrored in the transcriptome when the decline begins after exposure to nitrate (Cabeza et al. 2014). The analysis of the global response of nodule transcriptome after nitrate provision indicates that a shortage of ATP might be a cause of the decline by slowing down nitrogen incorporation and malate uptake of the symbiosome. However, rather than

inducing a more or less indirect reduction of the oxygen supply, or diversion of the assimilate flow away from the nodule, the nitrate ion seems to strike at the very heart of the N_2 reduction process, ie the formation of the nitrogenase complex itself and ATP generation (Cabeza et al. 2014).

1.3 Nitrate Transporters

Eukaryotic nitrate (NO_3^-) transporter genes were first isolated in *Aspergillus nidulans* and *Arabidopsis thaliana*; while both genes possessed 12 putative trans-membrane domains, the two genes were phylogenetically unrelated. (Unkles et al. 1991; Tsay et al. 1993) Ultimately, those two different genes would come to define the different types of transporters that mediate the uptake of nitrate from external sources, becoming known as the NRT1 and NRT2 families. Both family members transport nitrate together with a proton (H^+) in a symport mechanism that is driven by the pH gradients across membranes. Those proteins are involved in the control of nitrate flux from soil to root tissues and its allocation throughout the whole plant tissue (Miller et al. 2007).

Plant response to low or high NO_3^- conditions occurs at the level of plant roots and depend upon two different uptake systems: the high-affinity transport system – HATS – and the low-affinity transport system – LATS. The two families of nitrate transporters, NRT1 and NRT2, respectively contribute to LATS and HATS. There are two reported exceptions, proteins that display a dual HATS/LATS nitrate uptake activity and are thus involved in the uptake of NO_3^- at both high and low concentrations: *the Arabidopsis thaliana*'s AtNRT1.1 and the *Medicago truncala* MtNRT1.3. (Liu and Tsay 2003; Morere-Le Paven et al. 2011) , although recently the high affinity action of AtNRT1.1 *in planta* was not confirmed as it appears to be confined to the experimental system constituted by Xenopus oocytes (Glass and Kotur 2013).

NRT1 members share sequence similarity with peptide transporters (PTR), forming a superfamily that includes 53 members in *A. thaliana* and 80 in rice. Specific transporters for members of this superfamily cannot be argued by sequence data alone, and require an exhaustive biochemical approach. Several reports indicate that this superfamily contain members with the capacity to transport substrates as different as nitrate, di/tri-peptides, amino acids, glucosinolates, malate, auxin and ABA; some of its members even displayed

dual transport capacity for different substrates (Liu et al. 1999). Faced with this transport diversity, a new nomenclature was devised, the family renamed the nitrate peptide transporter family (NPF) and its members classified on the basis on their phylogenetic relationship; proteins from 33 fully sequenced plant genomes were analyzed and eight unambiguous clades were identified. Therefore, any given NPF member can be identified by a two numbers code indicating subfamily and relative position within said subfamily (Leran et al. 2014).

NRT2 proteins form smaller families of plant transporters, including 7 members in *Arabidopsis thaliana* and 4 members in rice. Unlike NPF genes, who have a great diversity of subtracts and their function cannot be so easily deduced, NRT2 members can be identified by sequence data and all the NRT2 proteins reported in higher plants have only nitrate as subtract. The NRT2 proteins do not have independent function; while the original *Aspergillus nidulans* NRT2 protein is functional on its own; in plants NRT2 require an additional component – NAR2/NRT3 – to perform nitrate transport activity. Biochemical transport analysis confirm that isolated NRT2 and NAR2 do not transport nitrate, while verifying a proton-dependent nitrate uptake when the two proteins are present (Tsay et al. 2007).

NPF and NRT2 proteins display homology with various families of membrane proteins that are ubiquitously present across all major kingdoms of life (in bacteria, fungi and animals). In these organisms, several family names are used to describe these proteins: POT (Proton-coupled Oligopeptide Transporter (POT), PepT/PTR (Peptide Transporter) and SLC15 (Solute Carrier 15) (Fei et al. 1994; Hauser et al. 2001; Daniel et al. 2006; Newstead et al. 2011). These membrane proteins display a predicted conserved structural arrangement of 12 transmembrane domains connected by short peptide loops, similar to the putative transmembrane domains of NPF and NRT2 members; the 3D arrangement of these domains was recently characterized for two bacterial homologs – PepTSo and PepTSt (Newstead et al. 2011; Solcan et al. 2012). In non-plant organisms, these proteins often display high substrate selectivity, with most being involved in nitrogen acquisition in the form of di- and tri-peptides; for example, the human PepT1 and PepT2 proteins are involved in dietary nitrogen uptake and the transport of β–lactam antibiotics (Meredith 2009).

1.3.1 Nitrate Transporters in *Arabidopsis thaliana*

The first transporter of the NPF family was reported in *Arabidopsis thaliana*, and the majority of the reported family members were characterized in this model plant (Miller et al. 2007). The original NPF, AtNRT1.1 or CHL1, is responsible for sensitivity to the herbicide chlorate and was identified as a capable to transport nitrate in specific assays (Tsay et al. 1993; Wang et al. 1998; Liu et al. 1999). Nitrate and/or di/tri-peptides transport capacity has been reported to eighteen different *Arabidopsis thaliana* NPF members, as well as affinity for other substrates such as auxin, ABA, and glucosinolates. AtNPF2.7, AtNPF2.9, AtNPF2.10, AtNPF2.11, AtNPF2.12, AtNPF2.13, AtNPF3.1, AtNPF4.6, AtNPF5.13, AtNPF5.14, AtNPF6.2, AtNPF6.3, AtNPF6.4, AtNPF7.2 and AtNPF7.3 have all been identified as nitrate transporters; AtNPF4.1 as an ABA transporter while AtNPF5.2, AtNPF8.1, AtNPF8.2 and AtNPF8.3 have been found to transport di-peptides. Various members of this family in Arabidopsis have been found to have dual transport activity: AtNPF2.9, AtNPF2.10 and AtNPF2.11 can transport glucosinolates in addition to nitrate; AtNPF4.6 transports ABA in addition to nitrate and AtNPF6.3 transports IAA. (Okamoto et al. 2003; Chiang et al. 2004; Segonzac et al. 2007; Almagro et al. 2008; Komarova et al. 2008; Fan et al. 2009; Li et al. 2010; Wang and Tsay 2011; Chen et al. 2012; Kanno et al. 2012; Nour-Eldin et al. 2012; Weichert et al. 2012). The majority of these reported NPF transport mechanisms involve a proton-coupled mechanism (Liu et al. 1999; Chiang et al. 2004)

NRT2 members in *Arabidopsis thaliana* display the aforementioned nitrate transporter capacity when forming a complex with NAR2/NRT3. Four out of seven NRT2 in Arabidopsis show a nitrate-related phenotype when mutated, showing reduced uptake or accumulation of nitrate in different tissues - NRT2.1, NRT2.2, NRT2.4 and NRT2.7 (Chopin et al. 2007; Li et al. 2007; Kiba et al. 2012). NRT2 family members might also have additional roles in *Arabidopsis thaliana*; AtNRT2.1 and AtNRT2.6 may be involved in the plant response to bacterial pathogen infection, as plants of *nrt2.1* and *nrt2.6* mutants show a reduced susceptibility to *Pseudomonas syringae pv* tomato and *Erwinia amylovora* bacteria. These NRT2 family members' negative impact upon bacterial resistance might be due to their role in down-regulating biotic stress defense mechanisms and favoring abiotic stress responses (Camanes et al. 2012; Dechorgnat et al. 2012).

1.3.2 Nitrate Transporters in legumes; *Lotus japonicus*

Sequence retrieval of NPF sequences from the *L. japonicus* whole-genome sequence resource (http://www.kazusa.or.jp/lotus/) allowed the identification of thirty-seven putative members; the results of this analysis have already been published. (Sato et al. 2008; Criscuolo et al. 2012) Sequence analysis predicted the NPF-defining, conserved structural arrangement of 12 transmembrane domains connected by short peptide loops in almost of the *L. japonicus* NPF members (Table 1).

A reiterated search led to the identification of an additional complete NPF sequence and thirty-tree un-completed unique sequences of predicted NPF genes, raising the total size of the *L. japonicus* NPF family to around 70 members. 51 out of these 71 members have been physically mapped on the Lotus genome, with there being a wide distribution of NPFs over the six chromosomes; sixteen of them are found on the chromosome 1, fifteen of them on chromosome 2 and other fifteen on chromosome 4, a single gene is located in chromosome 3 and two genes are found both in chromosome 5 and 6. There seem to be at least seven gene clusters that co-localize to same contigs with paralogous genes and short intergenic regions (Criscuolo et al. 2012).

The assignment of the 38 complete *L. japonicus* members according to the new NPF superfamily nomenclature, (Leran et al. 2014) assigning them to the 8 clades and their relative position, was based on BLAST analysis; each of the *L. japonicus* proteins was queried against already assigned NPF members of the *A. thaliana* and *M. truncatula* families, resulting in the nomenclature for the provisional list of LjNPF members indicated in Table 1. *L. japonicus* NPF members are distributed across all the 8 subfamilies (Table 2), being more prevalent in clade 6 (8 members) and the least prevalent in clade 1 (2 members).

The transcription of NPF1 and NRT2 plant genes is reported to be regulated by diverse factors as nitrate, nitrite, ammonium, glutamine, N starvation, light, sucrose, diurnal rhythm and/or pH (Wang et al. 2012); in many cases the transcriptional regulation is linked to a modulation of the nitrate uptake activities. A molecular characterization showing the expression profiles of a subset of *L. japonicus* NPF and NRT2 genes after exposure to different abiotic and biotic signals (e.g. nitrate, auxin, cytokinin, rhizobium) was reported by Criscuolo et al. (2012). This set of data can be integrated by the large amount of data reported in genome-wide analysis comparing expression profiles in inoculated and un-inoculated plants (Colebatch et al. 2004; Kouchi et al. 2004; Hogslund et al. 2009). Of

particular interest was the querying of the large set of *L. japonicus* transcriptome data encompassing different organs, stages of symbiotic interaction and root nodules development in wild type and mutant genotypes by Hogslund et al. 2009. A large number of Lotus NPF and NRT2 genes are identified by the probesets exploited in this GeneChip approach and the profiles of expression in root and young mature nodules are reported in table 1 (Hogslund et al. 2009; Takanashi et al. 2012). It is worth pointing out that seven members of this family cited above show a clear-cut induction profile in nodular tissue; moreover, the level of expression of these nodule-induced genes is not dependent on nitrogen fixation, as it is not affected in the *fix*- nodules obtained from the *sen1* and *sst1* mutants (Hogslund et al. 2009). This data has been updated by the analysis reported by Takanashi et al. (2012), indicating that for 5 out of 8 nodule-induced NPF members, the peak of transcription occurs in the infection zone of the nodule.

Table 1: List of complete LjNPF and LjNRT2 genes with indication of structural features of the predicted proteins (TM = transmembranar domains)

LjNPF old name/new name	**aa lenght**	Number of TM domains
chr3.LjT07H20.20/LjNPF1.1	585	12
chr4.CM0617.810/LjNPF1.2	576	12
chr4.CM0170.180/LjNPF2.1	579	12
chr2.CM0608.1290/LjNPF2.2	635	12
chr4.CM0170.40/LjNPF2.3	601	12
chr4.CM0170.210/LjNPF2.4	593	12
chr1.CM0147.130/LjNPF2.5	590	12
chr2.CM0903.350/LjNPF3.1	580	12
chr1.CM1911.210	616	11
chr1.CM1911.220	599	11
chr2.CM0608.1210/LjNPF4.1	581	12
chr4.CM0170.290/LjNPF4.2	557	12
chr4.CM046.1690/LjNPF4.3	591	12
chr6.CM0118.580/LjNPF4.4	605	12
chr1.CM0017.480/LjNPF4.5	634	12
chr6.CM1625.50/LjNPF5.1	587	11
chr1.CM0295.1000/LjNPF5.2	592	12
chr1.CM0295.980/LjNPF5.3	606	12
chr1.CM0295.970/LjNPF5.4	608	12
chr2.CM0081.1270/LjNPF5.5	539	12
chr1.CM0125.390/LjNPF5.6	573	12
chr1.CM0017.480/LjNPF6.1	634	12
chr2.CM0826.350/LjNPF6.2	583	12
chr2.CM0021.3040/LjNPF6.3	613	12
chr4.LjB20H09.30/LjNPF6.4	593	12
chr2.CM0826.370/LjNPF6.5	603	12
chr2.CM0545.330/LjNPF6.6	581	12
chr2.CM0021.2180/LjNPF6.7	598	12
chr2.CM0021.2200/LjNPF6.8	582	12
chr1.CM0017.770/LjNPF7.1	603	13
chr4.CM0247.130/LjNPF7.2	594	12
chr1.CM0141.10/LjNPF7.3	582	12
LjT24M05.60/LjNPF8.1	570	11
chr4.CM0026.890/LjNPF8.2	586	11
chr4.CM0026.930/LjNPF8.3	586	12
chr4.CM0026.860/LjNPF8.4	566	12
chr4.CM0026.870/LjNPF8.5	591	12
chr2.LjT15I01.230/LjNPF8.6	570	11
chr4.CM0026.880/LjNPF8.7	579	12
LjNRT2		
chr1CM0001.20	459	12
chr3.CM0649.30/LjNRT2.1	531	12
chr3.CM0649.40/LjNRT2.2	531	12
chr4.CM0161.180	508	12

Table 2: Sub-classification of the 38 *L. japonicus* NPF proteins in the eight sub-clades identified by Leran et al. (2013). The *A. thaliana* NPF members are included for comparison as well as the dual *M. truncatula* nitrate transporter MtNRT1.3 (in italic). Clade numbers indicate the different subfamilies. When known, the transported substrates are indicated in brackets.

Clade	*L. japonicus* Locus id.	New name	*A. thaliana/old name*
1	chr3.JjT0H20.20	LjNPF1.1	AtNPF1.1
	chr4.CM0617.810	LjNPF1.2	AtNPF1.2/AtNRT1.11 (nitrate)

			AtNPF1.3
2	chr4.CM0170.180	LjNPF2.1	AtNPF2.1
	chr2.CM0608.1290	LjNPF2.2	AtNPF2.2
	chr4.CM0170.40	LjNPF2.3	AtNPF2.3
	chr4.CM0170.210	LjNPF2.4	AtNPF2.4
	chr1.CM0147.130	LjNPF2.5	AtNPF2.5
			AtNPF2.6
			AtNPF2.7/AtNAXT1 (nitrate)
			AtNPF2.8
			AtNPF2.9/AtNRT1.9 (nitrate; glucosinolate)
			AtNPF2.10/AtGTR1(glucosinolate)
			AtNPF2.11/AtNRT1.10 (nitrate; glucosinate)
			AtNPF2.12/AtNRT1.6 (nitrate)
			AtNPF2.1/AtNRT1.7 (nitrate; glucosinate)
			AtNPF2.14
3	chr2.CM0903.350	LjNPF3.1	AtNPF3.1/AtNitr (nitrate)
	chr1.CM1911.210	LjNPF3.2	
	chr1.CM1911.220	LjNPF3.3	
4	chr2.CM0608.1210	LjNPF4.1	AtNPF4.1/AtAIT3 (ABA)
	chr4.CM0170.290	LjNPF4.2	AtNPF4.2/AtAIT4
	chr4.CM0046.1690	LjNPF4.3	AtNPF4.3/AtNRT1.14
	chr6.CM0118.580	LjNPF4.4	AtNPF4.4/AtNRT1.13
	chr1.CM0017.480	LjNPF4.5	AtNPF4.5/AtAIT2
			AtNPF4.6/AtNRT1.2/AIT1 (nitrate; ABA)
			AtNPF4.7
5	chr6.CM1625.50	LjNPF5.1	AtNPF5.1
	chr1.CM0295.1000	LjNPF.2	AtNPF5.2/AtPTR3 (di-tripepeptide)
	chr1.CM0295.980	LjNPF5.3	AtNPF5.3
	chr1.CM0295.970	LjNPF5.4	AtNPF5.4
	chr2.CM0081.1270	LjNPF5.5	AtNPF5.5
	chr1.CM0125.390	LjNPF5.6	AtNPF5.6
			AtNPF5.7
			AtNPF5.8
			AtNPF5.9
			AtNPF5.10
			AtNPF5.11
			AtNPF5.12
			AtNPF5.13/AtNRT1.16 (nitrate)
			AtNPF5.14/AtNRT1.15
			AtNPF5.15
			AtNPF5.16
6	chr1.CM0017.480	LjNPF6.1	AtNPF6.1
	chr2.CM0826.350	LjNPF6.2	AtNPF6.2/AtNRT1.4 (nitrate)
	chr2.CM0021.3040	LjNPF6.3	AtNPF6.3/AtNRT1.1 (nitrate; auxine)

	chr4.LjB20H09.30	LjNPF6.4	AtNPF6.4/AtNRT1.3 (nitrate)
	chr2.CM0826.370	LjNPF6.5	
	chr2.CM0545.330	LjNPF6.6	
	chr2.CM0021.2180	LjNPF6.7	
	chr2.CM0021.2200	LjNPF6.8	*MtNRT1.3 (nitrate)*
7	chr1.CM0017.770	LjNPF7.1	AtNPF7.1
	chr4.CM0247.130	LjNPF7.2	AtNPF7.2/AtNRT1.8 (nitrate)
	chr1.CM0141.10	LjNPF7.3	AtNPF7.3/AtNRT1.5 (nitrate)
8	Lj24M05.60	LjNPF8.1	AtNPF8.1/AtPTR1 (di-tripep; histidine)
	chr4.CM0026.890	LjNPF8.2	
	chr4.CM0026.930	LjNPF8.3	
	chr4.CM0026.860	LjNPF8.4	
	chr4.CM0026.870	LjNPF8.5	
	chr2.LjT15I01.230	LjNPF8.6	
	chr4.CM0026.880	LjNPF8.7	

Amongst the NRT2 members of *L. japonicus*, molecular characterization revealed a single gene (chr1.CM0001.20) that was strongly induced in young and mature nodules (Criscuolo et al. 2012). On table X are also indicated the expression profiles of LjNRT2.1 and LjNRT2.2 genes exported from the data reported by Hogslund at al. (2009), which show a significant down-regulation in young and mature nodules. The data for LjNRT2.1 is consistent with the later report in Criscuolo at al. (2012); this is not entirely accurate for LjNRT2.2, in which only a slightly decrease of the transcript levels has been reported.

1.4 Other gene categories involved in the plant response to N stress: transcription factors

The crucial role of Nitrogen, which is taken up as nitrate by the roots of most high order plants, as an essential micronutrient for plant growth and development has been already stressed above. Although the regulation of nitrate acquisition and utilization has been well described at the physiological level, not too much is known of the molecular mechanisms and molecular players that governs nitrate responses in higher plants (Walch-Liu et al. 2005). Microarray analysis has been used to identify N-responsive genes in different plants. Most of the studies were performed on *A. thaliana* to analyze the molecular basis of the plant response to nitrate and were designed to determine the profiles of gene expression after plants are shifted to different nitrate conditions (Wang et al. 2000; Price et al. 2004; Scheible et al. 2004). As expected these kinds of studies indicated the potential

crucial roles of transcription factors in this response mechanism. However, only a few transcription factors (ANR1, NLP7, LBD37/38/39, SPL9) have been implicated in the regulation of gene expression by nitrate and most of these factors are encoded by genes that are themselves regulated by nitrate at the transcriptional level (Zhang and Forde 1998; Castaings et al. 2009; Rubin et al. 2009; Krouk et al. 2010). One exception is NIN-like Protein7 (NLP7), a putative transcription factor that is expressed constitutively and shares homology with NIN, an essential protein for nodulation in legumes (see above) that is regulated in *A. thaliana* at the post-transcriptional level (protein localization). ANR1, an Arabidopsis MADS-box gene, has been shown to promote lateral root growth on nitrate-rich patches (Zhang and Forde 1998). In the case of LBD TF, the overexpression of LBD37/38/39 factors represses the expressions of nitrate transporter and NR genes in Arabidopsis (Rubin et al. 2009).

1.4.1 The AP2/ERF transcription factor family

AP2 (APETALA2) and ERF (ethylene-responsive element binding factor) are the prototypic members of a family of transcription factors unique to plants, whose distinguishing characteristic is that they contain the so-called AP2 DNA-binding domain. AP2/ REBP genes form a large multigene family, and they play a variety of roles throughout the plant life cycle: from being key regulators of several developmental processes, like floral organ identity determination or control of leaf epidermal cell identity, to forming part of the mechanisms used by plants to respond to various types of biotic and environmental stress (Riechmann and Meyerowitz 1998). They AP2/ERF family can be divided into four major subfamilies of transcription factors: AP2, RAV, ERF and DREB (dehydration-responsive element-binding protein). Of these, many stress-inducible DREB subfamily members have been isolated and characterized. It has been established that they are major factors involved in plant abiotic stress responses by regulating gene expression via the DRE/CRT element (cis-acting dehydration- responsive element/C-repeat). The ERF subfamily members have also been reported to have binding capacity to the ERE motifs (ethylene-responsive element) in abiotic stress conditions.

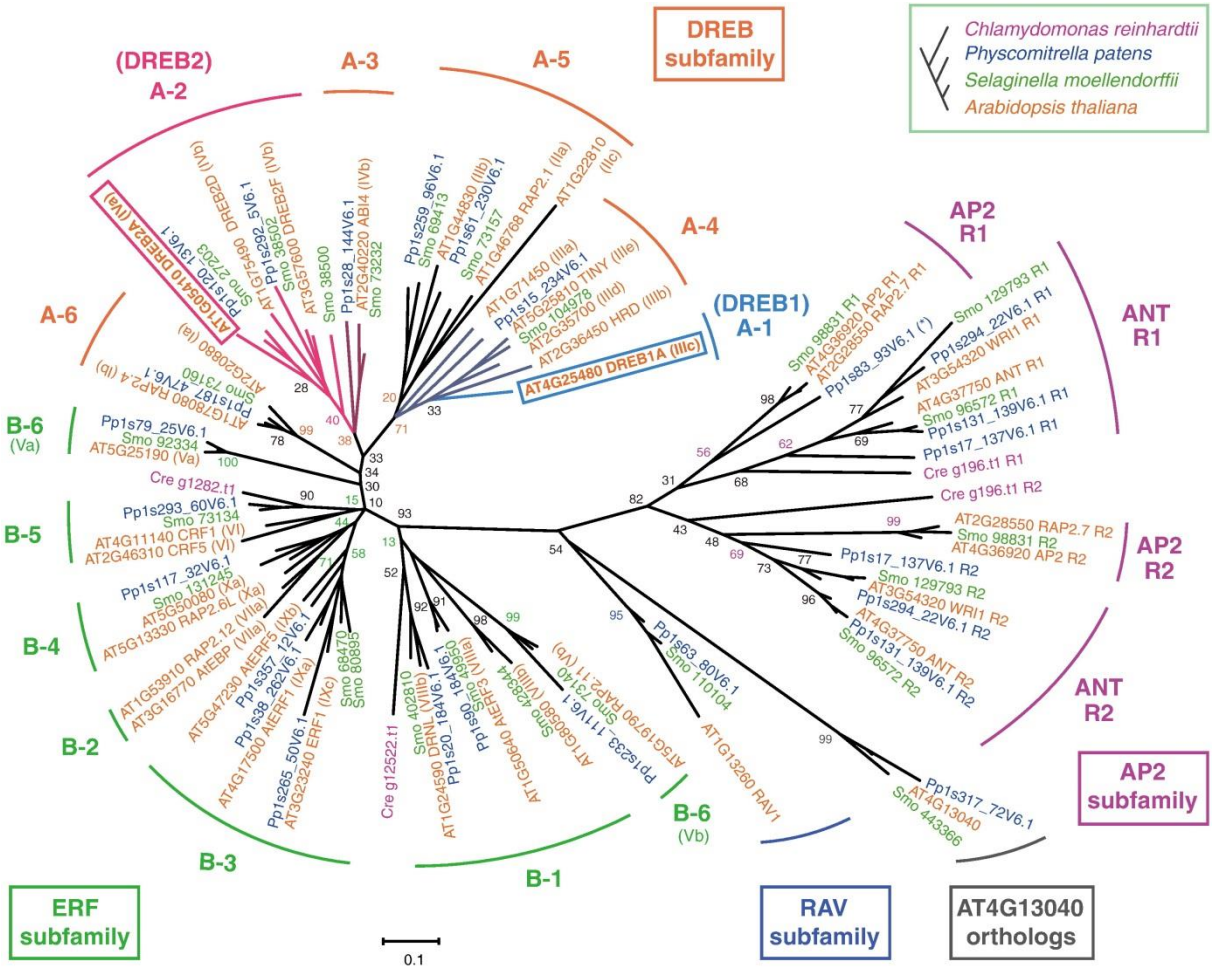


Fig. 14: Phylogenetic tree of ARP2/ERP transcription factors in green plants (Mizoi et al. 2012).

The AP2/ERF family is a large group of transcription factors containing AP2/ERF-type DNA binding domains, and family members are encoded by 145 loci in Arabidopsis and 167 loci in rice (Sakuma et al. 2002; Sharoni et al. 2011). This domain was first found in the Arabidopsis homeotic gene APETALA 2, and a similar domain was found in tobacco ethylene-responsive element binding proteins (EREBPs); these domains are closely related, suggesting they were conserved across higher plants, with the lowest plant in which an AP2/ERF family protein has been found being the green alga *Chlamydomonas reinhardtii* (Jofuku et al. 1994; Ohme-Takagi and Shinshi 1995; Weigel 1995; Shigyo et al. 2006). Sequences that are homologous to the AP2/ERF domain have been found in bacterial and viral endonucleases, and thus, it has been hypothesized that the AP2/ERF domain was transferred from cyanobacteria by endosymbiosis or from bacteria or viruses through lateral gene transfer events (Magnani et al. 2004). An NMR-based structural analysis of the Arabidopsis ERF1 protein in complex with a target DNA molecule revealed that the AP2/ERF domain contains

an N-terminal, three-strand β-sheet that recognizes a target sequence, as well as a C-terminal α-helix (Allen et al. 1998).

1.4.2 The AP2/ERF transcription factors in Arabidopsis

The RD29A/COR78/LTI78 gene of *Arabidopsis thaliana* has been reported to be induced by exogenously applied abscisic acid (ABA) and stress stimuli such as cold, dehydration and high salinity; RD92A stress-induced expression was maintained even in mutants deficient in ABA biosynthesis or signaling. This suggests that RD29A is regulated in both ABA-dependent and ABA-independent pathways. Analyses of the RD29A promoter reveal that the ABA-dependent expression of the RD29A gene is regulated by an ABA-responsive element (ABRE), whereas ABA-independent RD29A expression is regulated by a novel cis-acting element, TACCGACAT, which was named dehydration-responsive element (DRE). Similar cis-acting elements, a C-repeat (CRT) and a low-temperature-responsive element (LTRE), are also found in cold-inducible promoters; A/GCCGAC, which is a core DRE sequence, is shared among these cis-acting elements and is conserved in promoters of abiotic-stress responsive genes (Yamaguchi-Shinozaki and Shinozaki 1992; Yamaguchi-Shinozaki and Shinozaki 1993; Baker et al. 1994; Jiang et al. 1996)

Arabidopsis was also found to possess transcription factors that bind to these cis-acting elements: DREB1/CBF (DRE-binding protein 1/c-repeated binding factor) and DREB2. These proteins belong to the AP2/ERF family of transcription factors and activate gene transcription via specific binding to the DRE/CRT sequence in their promoters. DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 genes, which lie in tandem in the Arabidopsis genome, are induced by cold but not by dehydration or high salinity. Overexpression ofDREB1s/CBFs induces the expression of downstream stress-responsive genes and improves freezing, drought and high salinity tolerance in Arabidopsis. By contrast, DREB2A and DREB2B are not induced by cold but are induced by dehydration, high salinity and heat shock. Overexpression of a constitutive active form of DREB2A induces expression of dehydration- or heat shock-inducible genes and improves the drought, high salinity and heat shock tolerance of Arabidopsis. These findings indicate that DREB1 and DREB2 are involved in stress-induced acclimation processes in Arabidopsis by activating transcription

via binding to the DRE/CRT sequences in the promoters of stress-responsive target genes (Gilmour et al. 1998; Liu et al. 1998).

The DREB1 subgroup of Arabidopsis is a major regulator of cold-stress response. DREB1A/CBF3,DREB1B/CBF1 and DREB1C/CBF2 are rapidly induced in response to cold stress and Arabidopsis plants constitutively overexpressing any one of these genes display significant improvement in their tolerant to freezing, drought and high salinity, while presenting a reduced tolerant when their transcription is suppressed. Transcriptome analysis of DREB1/CBF-overexpressing Arabidopsis revealed that many cold-inducible genes are upregulated in these plants. These potential target genes encode cold-inducible proteins that function in survival at low temperatures including late embryogenesis abundant (LEA) proteins (i.e., hydrophilic proteins abundantly expressed during seed maturation and in response to cold or dehydration) and enzymes for sugar metabolism and fatty acid desaturation, as well as several transcription factors, suggesting the existence of downstream signaling pathways (Sakuma et al. 2002; Maruyama et al. 2004; Hannah et al. 2006). It is clear that DREB1/CBF genes play central roles in the survival of Arabidopsis at low temperatures by regulating cold-induced transcriptomic and metabolomic changes (Fig. 15). Transcription of DREB1s/CBFs is regulated by low temperature signals and circadian clock; non-cold-inducible DREB1s/CBFs still contribute in response against other abiotic stress responses (Mizoi et al. 2012).

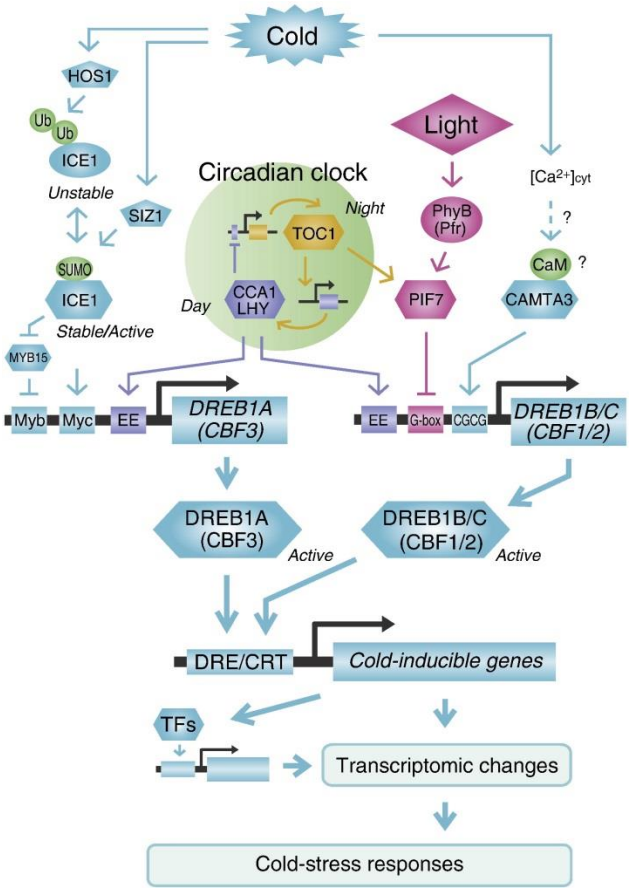


Fig. 15: DREB1 subfamily signaling pathway and its regulation in Arabidopsis. The cold-induced DREB1 are mainly regulated at the transcriptional level. The transcription of DREB1s/CBFs is under the control of low-temperature signals and circadian/light signals. Expressed DREB1/CBF proteins bind to DRE/CRT and activate transcription of target cold-inducible genes, including transcription factors, thus leading to transcriptomic changes, which eventually cause cold stress responses. Ub, ubiquitin; CaM, calmodulin; Myb, Myb-recognition sequence; MYC, MYC-recognition sequence; EE, evening element; CGCG, CGCG-Box; TFs, transcription factors (Mizoi et al. 2012).

The DREB2 subgroup has been reported to be involved in dehydration and heat shock responses. It is constituted of eight members in Arabidopsis and five in rice; DREB2 genes from grass species also seem to be involved in cold stress response (Sakuma et al. 2002; Matsukura et al. 2010). The DREB2 genes are further grouped in three subtypes through phylogenetic analysis; only subtype 1 genes are involved in stress response, while for genes of the subtype 2 and 3 roles their roles are unknown. In Arabidopsis, DREB2A and DRE2B seem to be the major DREB2s involved in dehydration-inducible gene expression through DRE/CRT in an ABA-independent pathway, being induced by dehydration, high salinity and heat (Fig. 16). Transgenic plants constitutively expressing DREB2A CA display stunted growth and improved tolerance to drought and high salinity but not to freezing. Many dehydration-

responsive genes, such as those encoding LEA proteins, are induced in these transgenic plants. In addition, many heat shock-responsive genes are also upregulated, and indeed, the transgenic plants exhibit enhanced tolerance to heat shock. Partial reduction of dehydration- or heat shock-responsive gene expression in dreb2a mutants indicates that DREB2A plays important roles in gene expression in response to dehydration and heat shock (Sakuma et al. 2006).

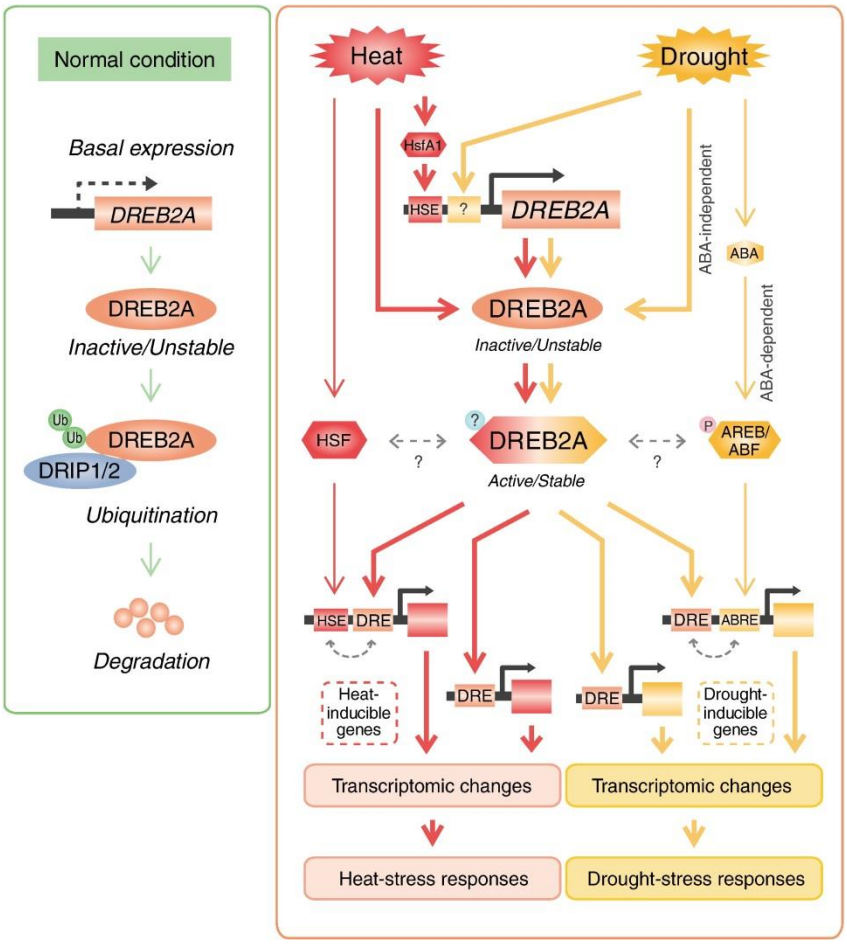


Fig. 16: DREB2 subfamily signaling pathway in Arabidopsis. The activity of DREB2A is regulated at both transcriptional and post-translational levels. Under normal conditions, the DREB2A protein expressed at a basal level is degraded via the ubiquitin-proteasome pathway. The transcription of the DREB2A gene is activated independently by heat shock or dehydration. The expressed DREB2A protein is further stabilized and/or activated by stress signals. Although the mechanism for this post-translational regulation is unclear, protein modifications may be involved in this process. DREB2A activates expression of target genes in a stress-specific manner via DRE/CRT sequences in the promoters. Heat- and drought-inducible genes' promoters often contain heat shock elements (HSEs) and ABA-responsive elements (ABREs), respectively (Mizoi et al. 2012).

The ABI4 (A-3) subgroup is related to the DREB1 subgroup but has distinct functions. The group is constituted by ABI4 and its orthologues and is reported to be involved in ABA, sugar and retrograde signaling (Koussevitzky et al. 2007; Matsukura et al. 2010). The A-4 subgroup is related to DREB1s/CBFs, sharing a conserved motif, but A-4 genes generally do not show high stress induction; overexpression of two genes (TINY and HARDY) results in stunted growth and thick leaves, while in the case of HARDY, also changing root architecture and conferring drought resistance; the functions of A-4 genes may not be clear, but this suggests they might play several roles in configuring stress responses (Nakano et al. 2006; Karaba et al. 2007).

The A-5 subgroup contains stress-inducible genes in Arabidopsis, cotton and soybean; in this subgroup there is a clade of proteins that have a functional ERF-associated amphiphilic repression (EAR) motif and genes encoding these EAR motif proteins are upregulated in transgenic plants over expressing DREB1 or DREB2 genes (Maruyama et al. 2004). Overexpression of these genes results in reduced expression of DREB1 or DREB2 target genes under stress conditions, while mutations in one of these proteins RAP 2.1 (Related To AP2.1) resulted in increased expression of these genes and in an improved tolerance to drought and freezing (Dong and Liu 2010). This suggests that EAR proteins of A-5 subgroup exert a negative feedback regulation downstream of the DREB1 or DREB2 pathways.

The A-6 subgroup also contains stress-inducible genes. RAP2.4 and RAP2.4B are expressed in response to dehydration, high salinity and heat. RAP2.4 and RAP2.4B are also responsive to cold and heat shocks, respectively; microarray analysis of a RAP2.4 overexpressor and a double mutant of RAP2.4 and RAP2.4B identified their targets as multiple aquaporines, suggesting a role in homeostasis. A *rap2.4a* mutant displays slightly lower expression of redox-regulated nuclear genes encoding chloroplast proteins and higher sensitivity to high light than the wild type. These findings suggest that A-6 proteins function in stress responses, but their target genes are different from those of DREB1s/CBFs and DREB2s (Lin et al. 2008; Rae et al. 2011). Analysis of both RAP2.4 overexpressing and silenced lines offered key phenotypes in Arabidopsis. A light-dependent phenotype, in which RAP2.4 overexpressing plants have slightly reduced apical hook curvature in darkness, and shorter hypocotyls when germinated in far-red, red and blue light; this suggests that RAP 2.4

acts as a light-dependent positive regulator of hypocotyl elongation, and it is shared by both the phytochrome and cryptochrome. RAP2.4 overexpressing seedlings growth in continuous red, far-red and blue light also possess smaller cotyledons; RAP2.4 overexpression also reduces induction of light-responsive genes involved in photosynthesis (CAB3 and RCBS), which is inverted in RAP2.4 silenced plants, suggesting a negative role of RAP2.4 in cotyledon expansion. RAP2.4 overexpressing plants also show earlier flowering on long-day (16h light/8h darkness) condition, but not in other light conditions. Hypocotyl elongation, cotyledon expansion, and gravitropic growth of hypocotyls are some of the plant development events that are regulated by ethylene; overexpressing RAP2.4 seedlings have reduced elongation of hypocotyls and are more agravitropic than their wild-type and silenced counterparts. These observations suggest that RAP2.4 plays an inhibitory role in ethylene-induced apical hook formation and negative gravitropism of hypocotyl growth, but promotes ethylene inhibition of hypocotyl elongation in dark-grown seedlings. The expression of a number of ethylene-responsive GCC-box containing genes, including PR3, PR4, and PDF1.2 increased after prolonged ACC treatment (100 µM ACC for 24 h), and this increase was higher in RAP2.4 overexpressing plants. RAP2.4 has also been implicated in drought-response, with RAP2.4 overexpressing plants remaining turgid even in conditions that withered wild-type and silenced RAP2.4 mutants; silenced, wild-type and overexpressing RAP2.4 plants also demonstrated normal ABA-induced stomatal closure and overexpression of genes containing DRE or CRT elements, suggesting RAP2.4 regulates drought stress in an ABA-independent way, through a pathway involving CRT/DRE elements (Lin et al. 2008).

ERF subfamily also seems to play an important role in abiotic stress responses. Submergence is a stress condition that inhibits the gas exchange of plants, thereby impairing photosynthesis and respiration; the process of acclimation to submergence includes the synthesis of ethylene. The rice ERT subfamily B-2 subgroup protein Sub1A (Submergence 1A) seems to confer tolerance to submergence, responds to submergence and ethylene and it negatively regulates cell elongation and carbohydrate consumption. Recently, Sub1A was shown to regulate the expression of other AP2/ERF family transcription factors during submergence: multiple genes in the B-1 and B-2 sugroups of the ERF subfamily and in the A-1 subgroup of the DREB subfamily, are upregulated by Sub1A, while a B-2 subgroup gene is

down-regulated. In deepwater rice, SK1 (SNORKEL1) and SK2, which are related to Sub1A, play central roles in ethylene –responsive internode elongation in response to submergence, regulating plant elongation in opposite ways to tolerate flash or long-term flooding (Fukao and Bailey-Serres 2004; Hattori et al. 2009; Jung et al. 2010). In Arabidopsis, RAP2.2 has been reported as being induced by hypoxia in an ethylene-dependent manner. Overexpression and knocking-out of the RAP2.2 gene results, respectively, in improved and impaired survival under hypoxia. Four transcription factors are downstream of RAP2.2: two (ERF71 and ERF73) are subgroup B-2 genes, and one (ERF4) is a subgroup B-6 gene; this implicates that a cascade of the B-2 subgroup and its downstream AP2/ERF genes are important for plant survival and hypoxia conditions (Hinz et al. 2010). Besides mediating ethylene-related responses, ERF subfamily includes members that are responsible for response to abiotic stresses such as drought and high salinity; while the exact function of those genes are mostly unknown, they are expected to regulate response to stress conditions in both ethylene-dependent and independent pathways (Mizoi et al. 2012).

1.4.3 The AP2/ERF transcription factors in legumes

Due to their association with ethylene signaling and ethylene-mediated factors, this family of transcription factors offers interesting candidates for actors of the regulation of nodule organogenesis.

The transcription of *L. japonicus* and *M. truncatula*, revealed through microarrays analysis the identification of a number of transcription factors whose expression changes early in the nodulation process (El Yahyaoui et al. 2004; Lohar et al. 2006; Asamizu et al. 2008). In addition to that, cis-regulatory elements have been reported to regulate Nod factor-dependent and epidermis-specific gene transcription; genetic analysis of the *MtENOD1*1 promoter resulted in the discovery of a Nod factor-responsive regulatory unit, denominated NF box, capable of directing Nod factor-elicited expression in root hairs. NF-box mediated expression requires a major GCC-like motif, which is also essential for the binding of root-hair specific nuclear factors; three closely-related member of the AP2/ERF transcription factors family (ERN1, ERN2, ERN3) can bind specifically to the NF box, and ERN1 and ERN2 are upregulated in root hairs after Nod factor treatment. Like their target gene MtENOD11, Nod factor-elicited activation of the ERN genes is dependent on the NFP locus,

an essential component of Nod factor perception and signaling in root hairs, as neither ERN1 or ERN2 where induced in nodulation defective nfp-2 mutants. Transient expression of Medicago ERN1, ERN2 and ERN3 in tobacco demonstrated that both ERN1 and ERN2 activate NF box-containing reporters, where ERN3 represses ERN1/ERN2-dependent transcription. This led to the creation of a model where regulation of Nod Factor-induced gene transcription in root hairs depends on the interplay of different activator and/or repressors ERNs (Amor et al. 2003; Arrighi et al. 2006; Andriankaja et al. 2007).

The work by Asamizu et al (2008) reported the identification of several transcription factors induced at different times upon infection with *M.loti* which are members of the CCAAT, bZIP, C2H2, Homeobox, NAC, WRKY, C3H, MADS, C2C2-Dof, CPP and AP2-EREBP families, the latter being the most represented (Table 3).

Table 3: Transcription factors induced post-nodulation in Lotus. Relative expression levels compared with uninfected root (Asamizu et al. 2008).

Gene Name	Accession No.	Transcription Factor Family	3 h	24 h	2N	4N	7N	12N
Group I (initial response)								
LjERF1	AB378626	AP2-EREBP	1.8	1.7	1.2	0.1	0.4	0.5
HDZ-M48	AB378627	Homeobox	1.8	1	0.7	0.1	0.1	0.1
CBF-A22	AB378628	CCAAT	1.6	1.3	1.1	0.3	0.5	0.2
bZIP-R91	AB378629	bZIP	1.9	1.1	0.9	0.2	0.3	0.9
LjRAP2.4	AB378630	AP2-EREBP	1.9	1.1	1.4	0.6	1.6	0.5
ZF-M39	AB378631	C2H2	1.8	1.1	0.8	1	1.2	0.8
LjERF2	AB378632	AP2-EREBP	1.7	1.3	0.6	0.8	1.4	1.6
Group II (induced at 2N)								
CBF-A01	AB378633	CCAAT	ND	ND	14.3	6.2	14.7	12.2
LjERF16	AB378634	AP2-EREBP	ND	ND	5.1	5.8	3.7	2.9
ZF-G96	AB378635	C2H2	ND	ND	2.7	3.1	1.5	0.7
LjERF18	AB378636	AP2-EREBP	ND	ND	1.6	15.4	25.2	11
LjWRKY30	AB378640	WRKY	ND	ND	1.8	1	2.7	1.8
Group III (induced at 4N)								
LjERF17	AB378637	AP2-EREBP	ND	ND	0.4	9.4	11.5	4.9
LjERFI9	AB378638	AP2-EREBP	ND	ND	1.2	1.7	3.1	4.7
NAM-A43	AB378639	NAC	ND	ND	1.3	1.8	2	2.3
Group IV (induced at 12N)								
RING-G83	AB378641	C3H	ND	ND	0.4	0.2	0.9	9.9
MADS-A18	AB378642	MADS	ND	ND	0.4	0.1	0.2	8
bZ1P-M43	AB378643	bZIP	ND	ND	1.1	0.2	0.5	2.1
Not clear								
Dof-M153	AB378644	C2C2-Dof	1.1	0.8	0.4	0.1	0.1	0.3
CPP-L56	AB378645	CPP	1.1	0.7	0.6	0.3	0.4	0.4

The abundance of AP2-EREBP transcription factors and their induction at different time points of nodulation suggested that they may play important roles in the regulation of the nodulation process, such as the ERN1-3 genes previously mentioned in Medicago. Amongst the *L. japonicus* proteins, orthologues to *A. thaliana* RAP2.4, ERF1, ERF2 were found. AP2-EREBP family members in Arabidopsis have been implicated in ethylene and/or jasmonic acid (JA) signaling, which have been reported to influence nodulation process;

Lotus AP2-EREBPs seem to have organ-specific responses to ethylene and jasmonic acid: LjERF2, LjRAP2.4, and LjERF18 were induced in both shoots and root. The induction ofLjERF2 and LjERF18 was observed in response to both ethylene and JA, and LjERF18 induction was higher in shoots than in root. The induction of LjRAP2.4 by ethylene and JA was observed in the root, and a synergistic effect was observed in shoots and root. A significant level of induction of LjERF1, LjERF19, and LjERF17 was observed only in the root. LjERF17 was preferentially induced by JA. A synergistic effect of ethylene and JA was observed for LjERF1 and LjERF19. LjERF1 exhibited synergistic up-regulation in response to ethylene and JA in a root-specific manner. LjERF1 was also functionally characterized and overexpressing hairy roots showed an increased number of nodules compared to wild-type, whereas suppression of LjERF1 expression caused a reduced in nodulation. ERF1 in Arabidopsis was reported to be involved in the activation of pathogen defense genes, which have to be suppressed during the nodulation process, so LjERF1 could be also involved in the regulation of pathogen defense. PR10-1 is a gene whose expression is induced by pathogens in Medicago and during establishment of symbiosis in Lotus, and thus, represents a reporter for the pathogen defense of legumes. Overexpression of LjERF1 did not affect the expression of LJPR10-1, while suppression of LjPR10-1 resulted in an induction of LjPR10-1 expression. As such, it is it is possible that LjERF1 is involved in the suppression of defense genes for the establishment of rhizobium infection (Asamizu et al. 2008).

1.2.4 LjRAP2.4

In the aforementioned work by Omrane et al. (2009) an affymetrix analysis in *L. japonicus* led to the identification of a pool of sequences whose expression was significantly up- or down-regulated by low vs high N conditions represented by 10 µM NH_4NO_3 and 10 mM NH_4NO_3 regimes, respectively. The chip analysis allowed to report general metabolic differences between Lotus plants grown in low-N and high-N conditions, with repression of genes involved in the nitrogen assimilation pathway suppressed in low-N, as well as genes involved in starch and sucrose synthesis and degradation; low-N plants also display increased expression of genes involved in amino acids ex novo synthesis and repression of those related to amino acid catabolism. Low-N conditions also showed an upregulation of phenylpropanoids and phenolics synthesis, as well as flavonoid metabolism, while other

biosynthesis pathways were down-regulated, implicating a shift from a primary to a secondary metabolism in nitrogen starvation. This establishes the metabolic changes between different nitrogen environments and its regulation (Omrane et al. 2009).

Amongst the genes upregulated and downregulated by low-N conditions are included transcription factors of various families: NAC, bZIP, GRAS and also AP2/EREBP. In particular, that chip analysis identified the orthologue of the *Arabidopsis thaliana* AtRAP2.4 gene, LjT01F24.60.nd as strongly up-regulated in plants pre-incubated for 10 days on low N vs plants pre-incubated on high N conditions (2.8 fold; Omrane et al. 2009). Interestingly, this LjT01F24.60.nd (hereafter called LjRAP2.4) up-regulation profile was also observed in plants pre-incubated on high N condition and then are shifted for nine days to low N permissive conditions. Strikingly, this nine days shift intentifed the range of time needed by Lotus plants to re-acquire the full competence for nodulation. This results together with the expression profile of LjRAP2.4 reported by Asamizu et al. (2008) and with the involvement of the AtRAP2.4 protein in the *A. thaliana* ethylene signaling pathway, suggested a very intriguing role of LjRAP2.4 as a factor playing a crucial role in the link between exogenous and endogenous nodulation regulatory factors.

2. Project Aims

2.1 Biochemical and functional characterization of *Lotus japonicus* NPF1 proteins

The nodulation process resulting from the symbiosis of legume roots and rhizobia allows legumes to be the major natural nitrogen-provider to the ecosystem, contributing roughly 200 million tons of nitrogen each year, the equivalent to over 150 billion Euro worth of fertilizer replacement value. Therefore, the substitution of legumes as bio-fertilizers for inorganic-N fertilizer might save non-renewable resources required to manufacture and distribute fertilizer offering some potential to mitigate environment pollution effects.

As sessile organisms, plants have developed sophisticated mechanisms to ensure appropriate adaptations to constantly changing environmental conditions. As an example, the uptake of nitrate from the soil is a critical process controlled by complex regulatory networks underlying either external or internal cues to modulate the uptake capacity in accordance with the plant nutrient demand and the nitrate availability in the soil. As described above, plant roots have two different uptake systems to cope with low or high NO_3^- concentrations in soil, the high affinity and low affinity NO_3^- uptake systems (HATS and LATS). Two types of NO_3^- transporters, known as the NPF and NRT2 families, contribute to LATS and HATS for both nitrate uptake and its distribution within the plant (Miller et al. 2007). A complete functional characterization has been recently carried out for several *A. thaliana* nitrate transporters indicating their involvement in the control of nitrate flux from soil to root tissues and throughout the whole plant body.

Plant adaptations include also the capacity to respond to changes of the nutrient availability in the soil by modulating their root system developmental plan. NO_3^- is able to trigger signalling pathways modulating systemically, or locally, lateral root development (Zhang & Forde 1998). In Arabidopsis, increasing evidence indicate that the NRT1.1 protein plays a dual nutrient transport/signalling role (transceptor) as major sensor of external nitrate concentrations. Recently, the reported NRT1.1 activity as an auxin transport facilitator permitted the definition of a mechanism connecting nitrate and auxin signalling, mediating secondary root elongation (Krouk et al. 2010).

The investigation of the mechanisms underlying the physiological link between N availability and nodule initiation, development and functioning has been the latest focus of our group. Nitrate availability in the soil is known to strongly affect nodule formation as low and high concentrations exert a positive and negative effect on initiation of the organogenesis process, respectively (Carroll & Mathews 1990; Barbulova et al. 2007). As for secondary root developmental control, the nitrate effect on nodulation is exerted through both a local and systemic control (Omrane & Chiurazzi 2009). The mechanisms and factors involved in both local and systemic N supply controls, as well as the potential targets of their action on the pathways leading to nodule initiation are almost completely unknown. Recently, CLE peptides have been reported to be involved in the auto-regulation mechanism controlling the nodule formation (Okamoto et al. 2009). A potential action of NRT proteins could be played by controlling the transport of nitrate and/or peptides involved in the control of nodule formation. Another possibility could be an involvement in the signalling pathways that senses and transduces the nitrate signal to the root machinery involved in the nodule organogenesis.

Phytohormones are primary regulators of secondary root development, but they also play major roles in nodule formation and development (Ding & Oldroyd 2009). Nod-factor secreted by rhizobia in the soil are perceived by root epidermis and axial and radial auxin transport, control the initiation of cortical cell divisions leading to nodule formation through a shift in the local auxin-to-cytokinin ratio. The crucial role of cytokinin in nodule organogenesis has been also confirmed through the identification of a cytokinin receptor whose role in the Nod factor transduction pathway has been recently demonstrated (Tirichine et al. 2007). Abscisic acid (ABA) is also involved in the control of epidermal responses to rhizobial inoculation with a negative action on the formation of infection structures (Suzuki et al. 2004). Therefore, a possible interplay between NRT proteins and nodule development could also pass through their involvement in the phytohormones pathways controlling this organogenesis process. In the cases of the *A. thaliana* NRT1.1 and NRT1.2 a direct auxin and abscisic acid uptake capacity has been recently reported (Krouk et al. 2010; Kanno et al. 2012).

The functioning of mature nodules is based on a complex network of nutrient exchanges between partners. In addition to transporting NO_3^-, dipeptides or tri-peptides,

auxin, and/or abscisic acid, members of the NFP1 have been found capable to transport amino acids (Waterworth & Bray, 2006) and dicarboxylic acids (Jeong et al., 2004). Besides, only a small number of NFP1 proteins have been functionally studied compared with the large number that exists in higher plants (53 in *A. thaliana*, 80 in rice); thus, the number of biochemical functions ascribed to this family may expand and the biochemical characterization of the *L. japonicus* NRT proteins can permit to identify specific roles linked to nodule functioning.

The first molecular characterization of NFP1 and NRT2 gene families in the model legume *Lotus japonicus* (Criscuolo et al. 2012) showed a repressible, inducible or constitutive response to provision of nitrate, auxin or cytokinin providing a sound foundation for future experiments aimed to elucidate the specific roles of each transporter. This analysis allowed also the identification of *L. japonicus* NFP1 and NRT2 genes specifically expressed during the symbiotic interaction with *M. loti*, offering us a range of candidates for actors involved in the signalling pathways involved in control of nodule organogenesis.

One of the aims of my work is the functional characterization of members of the NPF family in *Lotus japonicus* through a molecular genetic approach. This gene family was scarcely characterized in legume plants, and the impact of these transporters on the formation/development/activity of Nitrogen fixing nodules still remains an open question.

2.2 Molecular and functional characterization of Rap 2.4 transcription factor role in *Lotus japonicus* nitrogen-fixative symbiosis

Ethylene's role in regulating plant development has been well reported in Arabidopsis, being a promoter of root hair formation and elongation, as well as inducing auxin biosynthesis, leading to alterations of the auxin root tip gradient and the auxin-to-cytokinin ratio (Riechmann and Meyerowitz 1998). Analysis in legumes have left pretty clear that ethylene has a repressor effect upon nodulation, preventing calcium spiking and inducing an early arrest, all while regulating nodule primordial positioning (Lee and Larue 1992; Hunter 1993; Heidstra et al. 1997; Schmidt et al. 1999; Oldroyd et al. 2001).

With ethylene displaying such an important role in regulating both plant development and nodule organogenesis, there can be no doubt about the important of the

molecular actors involved in the regulation of ethylene signaling. The AP2/ERF transcription factor family is known for its ethylene-responsive binding domains, and its close relationship with ethylene and stress response has already been well characterized in Arabidopsis; bridging between the ethylene signal and actual stress response, AP2/ERF family members regulate the transcription of genes involved in adaptations to abiotic stress (Riechmann and Meyerowitz 1998; Mizoi et al. 2012). Subfamilies like DREB1 regulate response to cold, while DREB2 mediate stress responses to heat and dehydration, while others contribute to regulate DREB1 and DREB2 and regulate their response through negative feedback (A5) or have been considered as having a putative role meditating other stress responses (ABI-4/A-3) (Mizoi et al. 2012).

A particular family, A-6, has been reported to contain two stress-inducible genes, RAP2.4 and RAP2.4B. Their functional characterization underlying the role the role these proteins play in the ethylene signaling pathway in *A. thaliana* has been described above, in the Introduction section of this thesis (Lin et al. 2008; Mizoi et al. 2012).

With ethylene showing a clear cut nodulation-inhibiting phenotype, and RAP2.4 being involved in ethylene downstream signaling in Arabidopsis, a hypothesis presents itself: may orthologues of RAP2.4 in legumes be involved in mediating ethylene-dependent control of nodule organogenesis?

Transcriptome analysis of the legumes *L. japonicus* and *M. truncatula* allowed the identification of a number of transcription factors whose expression changes early in the nodulation process, which include various members of the AP2/ERF family, including orthologues to RAP2.4. The Lotus orthologue LjRAP2.4 was found to be expressed during the initial stages of nodulation, achieving its peak expression during the first 3 hours after infection with M.loti, and treatment with ethylene or jasmonic acid induced LjRAP2.4 expression (Asamizu et al. 2008).

The affymetrix analysis of Omrane et al. (2009) allowed the identification of sequences whose expression was up/down-regulated with low and high nitrogen conditions; LjRAP2.4 was among those genes, being induced in low nitrogen concentrations compared to high nitrogen and one plants shifted from high nitrogen to low nitrogen regimen.

Due to its induction by ethylene, it is of particular interest to confirm if LjRAP2.4 demonstrates ethylene-related phenotypes similar to those reported for its Arabidopsis

orthologue: regulation of hypocotyl elongation, cotyledon expansion, and gravitropic growth of hypocotyls, activation of ethylene-dependent genes and regulation of drought conditions response. The induction in low nitrogen conditions and in the initial stages of inoculation suggests a role in the control of nodulation. To address the question whether LjRAP2.4 is involved in the *L. japonicus* ethylene-signalling pathway and if as such it plays a role in the regulation of the nodule organogenesis program, I followed a reverse-genetics approach and obtained transgenic plants for overexpression and RNA interference of the LjRAP2.4 target gene.

3. Material & Methods

3.1 Biological Material

The experiments reported in this doctorate work have been performed upon plants *Lotus japonicus* of the ecotype GIFU F129 wild type (wt), as well as stable overexpressing and silenced stable transformants pCAMBIA-LjRAP2.4 and pB7GWIWG2(II)-LjRAP2.4. In addition to wild type and stable transformant plants, we also used LORE1 – Lotus retrotransposon 1 – insertion mutagenesis lines generously provided by the *CARB* (Centre for Carbohydrate Recognition and Signalling) in Denmark (Urbanski et al. 2012).

The microbic partner is *Mesorhizobium meliloti* strain R7A carrying a Rifampicin resistance cassette integrated into the chromosomial DNA.

3.2 T-DNA construct preparations

The technical details for preparations of overexpressing and RNAi constructs for LjRAP2.4 as well as promoter-*gus*A fusions are given in the appropriate paragraphs of the Results section.

3.3 Sterilization of *Lotus japonicus* seeds

Before germination *Lotus japonicus* seeds are sterilized with 2.5% Sodium hypochlorite supplemented with 0.05% Triton-X100. Seeds are gently shacked into this solution for 20 min. Then they were washed with sterile H_2O under laminar horizontal flow and stored upside down at 4ºC in sterile water in dark for 24 hours. Thereafter, they are transferred on plant agar (1%) Petri dishes and put again at 4ºC in dark for 24 hours. The day after the Petri dishes were transferred in growth chamber at 23ºC for another 24 hours, covered with aluminum paper to maintain the darkness necessary for germination. Finally, roots of germinated seedlings are adjusted and the Petri dishes are positioned vertically in growth chambers at 23º C under 16 hours photoperiod and light intensity of 246 µE s-1 m-2.

3.3 Growth media preparation and in vitro growing conditions

Gamborg's B5 medium (B5): 3.04g/L of B5 powder (Duchefa).

Gamborg's B5/2 medium: 1.52g/L of B5 powder (Duchefa).

Murashige and Skoog MS medium: 4.3 g/L of MS powder (Duchefa)

Plant growth media is buffered by addition of 0.5 g/L of 2.4-Dichlorophenoxyacetic acid (MES). The final pH of 5.7 is adjusted by addition of KOH. Plant agar (Duchefa) is added at 1% concentration ithe solid media.

Antibiotics: To block the bacterial growth of *M.loti*, cefotaxime is used in the concentration of 0,2 mg/ml. For selection of transformed plants, hygromycin in the concentration of 5 γg/ml was used.

3.3. 1 Standard growth media composition

Gamborg's B5 medium (B5)

Micro-elements	**Concentration (mM)**	**Final concentration (µM)**
$CoCl_2 \cdot 6H2O$	0.025	0.11
$CuSO_4 \cdot 5H2O$	0.025	0.10
NaFeEDTA	36.70	0.10
H_3BO_3	3.00	48.52
KI	0.75	4.52
$MnSO4 \cdot H2O$	10.00	56.16
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.30
$ZnSO_4 \cdot 7H_2O$	2.00	6.96
Macro-elements	**Concentration (mM)**	**Final concentration (mM)**
$CaCl_2$	113.23	1.02
KH_2PO_4	2500.00	24.73
KNO_3	1900.00	18.79

$MgSO_4$	121.56	1.01
NH_4NO_3	130.44	1.09
$(NH_4)_2SO_4$	134.00	1.01

Vitamins	**Concentration (mM)**	**Final concentration**
Myo-Inositol	100.00	0.56 mM
Nicotinic Acid	1.00	8.12 µM
Pyridoxine - HCl	1.00	4.86 µM
Thiamine HCL	10.00	29.65 µM

Murashige and Skoog medium (MS)

Micro-elements	**concentration (mM)**	**Final Concentration (µM)**
$CoCl_2 \cdot 6H_2O$	0.025	0.11
$CuSO_4 \cdot 5H_2O$	0.025	0.10
NaFeEDTA	36.70	0.10
H_3BO_3	6.20	0.10
KI	0.83	5.00
$MnSO_4 \cdot H_2O$	16.90	0.10
$Na_2MoO_4 \cdot 2H_2O$	0.25	1.30
$ZnSO_4 \cdot 7H_2O$	8.60	29.91

Macro-elements	**Concentration (mM)**	**Final concentration (mM)**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61

Vitamins	**Concentration (mM)**	**Final concentration (µM)**
Nicotinic Acid	1.00	8.12
Pyridoxine - HCl	1.00	4.86

Thiamine HCL	10.00	29.65

B&D Medium

Elements	Concentration (µM)
$CoCl_2 \cdot 6H_2O$	0.2
$CuSO_4 \cdot 5H_2O$	0.4
Fe-Citrate	10
H_3BO_3	2
$MnSO_4 \cdot H_2O$	1
$Na_2MoO_4 \cdot 2H_2O$	0.2
$ZnSO_4 \cdot 7H_2O$	0.5
$CaCl_2$	1
KH_2PO_4	0.5
$MgSO_4$	0.25
$(NH_4)_2SO_4$	500

3.3. 2 Alternative nitrogen-sources growth media composition

For the preparation of modified growth media, in which the complete absence of a nitrate source is required, or the concentration of the nitrogen source is controlled, the KNO_3, NH_4NO_3 e $(NH_4)_2SO_4$ of the base BS/MS medium are replaced by KCl as alternative potassium source. This medium deprived of nitrogen is denominated –N. To this medium, different nitrogen sources at different concentrations can be added, allowing elaboration of different growth media, depending on the requirements of the experiment.

3.4 Hygromycin sensitivity/resistance assays

The seeds *L. japonicus* to be tested for the phenotype of resistance/sensibility to hygromycin are germinated in plates containing the culture medium B5/2 with hygromycin 5 γg/ml; as a negative control, wild plants are added to the same plates. After 2-3 weeks, the phenotype of the plants is compared with the sensibility phenotype: high accumulation of anthocyanins, undeveloped shoot and extremely small roots.

3.5 *In vitro* nodulation

To assure reproducible in vitro conditions when studying the diverse states of the symbiotic process of the legume *L. japonicus* and the bacterium *Mesorhizobium loti* it is important to have uniform infection conditions, in which each single radical meristem enters in contact with the same number of bacteria.

3.6 Preparation of bacteria

The chosen Rhizobia strain is inoculated from the -80ºC stock into 5 mL TYR ($CaCl_2$) supplemented with the appropriate antibiotic (Rifampicin for wild type strains) and let grow for 2 days (rotation) at 30ºC. Cells are harvested at 4000 rpm, 4ºC during 10 min and washed with PBS (1x) before to be centrifuged again (same settings). The optical density is checked at 600 nm, assuring an optimal concentration of 10^7cells/20µL per root tip is used.

3.7 Infection of plants with *M. loti*

Once a root system of optimal dimensions has been obtained, with roots with at least 1 cm of length, the plants are positioned over a filter and on a plate containing a nodulation-adequate media. Each individual root is inoculated with approximately 10^7cells, the bacterial suspension diffused across the entire length of the competent region. The filter paper is important to assure a proper, uniform diffusion, but has to be removed 4 days after infection. Infected roots are kept in the dark for the next 4 weeks of infection and the number of nodules is analyzed at the stereomicroscope.

3.8 *In vivo* nodulation

Since with the in vitro nodulation assay, Lotus plants might be kept in Petri dishes for a limited period of time, in some cases a slight defect of the nodule activity may need a longer incubation to test the symbiotic phenotype. This can be achieved by inoculation of Lotus plants with *Mesorhizobium loti*, in a closed-system of culture boxes (Magenta), where the substrate of growth is constituted by, a 1:1 mix of vermiculite and sand.

3.9 Preparation of bacteria

The chosen Rhizobia strain is inoculated from the -80ºC stock into 5 mL TYR ($CaCl_2$) supplemented with the appropriate antibiotic (Rifampicin for wild type strains) and let grow

for 2 days (rotation) at 30ºC. Cells were harvested at 4000 rpm, 4ºC during 10 min and washed with PBS (1x) before to be centrifuged again (same settings). The optical density was checked at 600 ηm, assuring an optimal concentration of 10^7cells/20µL is used.

3.10 Infection of plants with *M. loti*

Once a plant has achieved a complex root system and a long shoot with around 5 cm of length, the plants are moved to plant-growth boxes (Magenta). Each individual plant is inoculated at the bottom with approximately 10^{10} cells, the bacterial suspension being diffused across the vermiculite/sand mixture. The plants are kept closed in a growth chamber for 4 weeks of infection and the number of nodules is analyzed at the stereomicroscope.

3. 11 β-glucuronidase histochemical assay

The protocol is used to unravel the activity of the *gus*A cassette that provides a tissue localization and if desired a quantification of the promoter activity of the gene of interest. Entire seedlings or organ parts (shoots/roots parts) or even pieces of leaves coming from the transgenic plants expressing the GUS cassette are used. Those plant materials are placed into 2mL eppendorfs containing the GUS staining solution mix: Buffer Phosphate 100mM ($NaPO_4$) , pH 8.0, X-Glucorinidase 1mM (dissolved in dimethylformamid) and 0.01% Triton-100X. The eppendorfs are vacuum infiltrated for 10 minutes then the sample are incubated in dark over night at 37ºC. The day after the sample could be either taken to be analyzed under microscopy or let one more day in dark at 37ºC. To reveal weak coloration in shoot part, a post treatment of the sample with Ethanol 100% is necessary to extract the green color pigment chlorophyll.

3.12 Preparation of root section at vibratome and optical microscope observation

Root fragments (around 3mm) are embedded in agarose 6%. Once agarose solidifies, a square pyramid “block” is carved around the root/nodule inclusion, which is then used to obtain sections with 55-60 µm of thickness at the Leica VT 1000s vibratome. The settings used are blade movement speed: 2 and blade vibration frequency: 3. The thin agarose layer containing a section of rood and/or nodule, is immersed in phosphate buffer 50 mM and

analyzed at both Stereomicroscope Leica MZ16FA and LeicaDM6000 microscope. The objectives used are:

- 5x Hcx PL Fluotar NA 0.15
- 10x Hcx PL Fluotar NA 0.3
- 20x Hcx PL Fluotar NA 0.5
- 40x Hcx PL Anapo NA 0.75
- 100x Hcx PL ApoCS NA 1.4 (immersion oil)

3.13 Bacterial Transformation

3.13.1 Heat shock transformation:

Spin down the vials containing the DNA mix reaction (Ligase, plasmid, etc), meantime the Eppendorf with -80ºC frozen competent cells are thawed on ice for 15 min. The ligase or the construct of interest is added to the competent cells and let other 15 min on ice. The transformation mix is then transferred for 30s into a preheated water bath at 42ºC. The transformation mix was then immediately put on ice and 1mL of TY, LB or S.O.C. without antibiotic added. The mix is then transferred for 1 hour in a water bath preheated at 37ºC. Finally, cells are plated on the appropriate medium supplemented with the appropriate antibiotic.

3.13.2 Electro-transformation:

The vials containing the DNA reaction is briefly centrifuged. The electro-competent cells (Top 10 F') are thawed in ice during 15 min and the transformation cuvettes are also cooled down on ice. The cells supplemented with the clean DNA are keeping sterile conditions under a vertical laminar flow apparatus. Immediately after electroporation 1mL of SOC medium is added to the electroporated cells.

3.14 Plant Transformation-regeneration procedure mediated by *Agrobacterium* transformation

The flowchart is undertaken as follow:

1. Pre-culture: the roots must be enough long but still young in terms of tissue quality. A pre culture after germination of the seedlings during 10 days is necessary then root system are detached and incubated for 5 days on CIM medium to induce cell proliferation and make them enough thick. The CIM, callus induction medium induce callogenesis;

2. Infection: cut the roots in small pieces (0.5 cm) and let them soak 10 min into the *A. tumefaciens* liquid of culture and squeeze them from time to time using forceps;

3. Co-culture: transfer the explants on fresh CIM medium supplemented for two days.

4 Transfer the co-cultivated roots for two days on fresh CIM medium supplemented with 200 mg/L cefotaxime;

5. Wash the explants with sterile water and dry them on sterile paper before transfering them on selective CIM medium supplemented with 200 mg/L cefotaxime and 15 mg/L hygromicin and 3% sucrose;

6. Incubation: Incubate the plants in growth chamber (16h photoperiod; 23ºC) 3 to 4 week until green callus are formed. Generally the green callus is formed on the surface of the explants;

7. Transfer on SIM (shoot induction medium supplemented with 200mg/L Cefotaxime, 15mg/L Hygromicin, 0.5 mg/L TDZ (Thidiazuron phyto-hormone) and 3% sucrose) only the green calli that indicates the potential success of the transformation procedure since they are resistant to the Hygromicin by contrast to the rest of the white-necrotic untransformed tissues. This step requires 15 days before the rising of the shoot primordial. Then transfer the shoot on SIM medium containing 200 mg/L cefotaxime, 10 times less TDZ (0.05 mg/L) and 3% sucrose;

8. Transfer the shoot primordia on SEM supplemented with 200 mg/L cefotaxime and 1% sucrose by dissecting them carefully from the rest of the tissue. This step requires 15 days to obtain sufficient shoot lengths allowing their easy separation form the rest of the callus;

9. Shoots are transferred on RIM (Root induction medium). This consents the root organogenesis in the basal cut part of the shoot. 10 days are required to obtain roots;

10. The small regenerated potential transformants are transferred on REM (root elongation medium) for one more week before to be transferred in ground.

From the roots incubation to the regenerated transformants 3 months are needed and other 4 are usually necessary before the flowering and the first mature pods (containing T1 seeds) harvestings.

3.15 *Agrobacterium rhizogenes*-mediated transformation: Hairy root experimental system

Transformation of root systems through *Agrobacterium rhizogenes* occurs over a week, in order to produce a root system transformed with our construct of interest. This transformation is performed on 1 week-old seedlings, whose root system is still developing but has the required dimensions to allow the following procedure:

1. Grow the *Agrobacterium rhizogenes* strain from the frozen stab at -80ºC over-night in a 5 ml YEB medium inoculum at 30°C.

2. Recover strain DNA by mini-prep in order to check by restriction analysis the plasmid fidelity. After checking the strain, dilute 1:100 in a 20 ml YEB medium culture for a new over-night growth at 30ºC.

3. The transformation proper is performed Pour the 20 ml YEB culture in a sterile Petri dish. Cut the primary root of the Lotus seedlings with scissors (roots must be 1-1.5 cm long) a few millimeters bellow the hypocotyl/primary root border, right in the root proper. Immediately put the wounded part of the plant into the bacteria culture, if possible hooking the plant at the edge of the Petri dish by using the unfolded cotyledons. Leave the wounded part in contact with the bacteria culture for 3 minutes. Transfer the plants on B5/2 medium (0.8 % agar), plus 1% Sucrose plus vitamins. To facilitate this transfer, make a little hole into the medium with forceps and put the wounded root into the hole and leave the cotyledons in contact with the air. Seal with parafilm and put the plants in a 23ºC growth chamber for two days.

4. Two days later, take the plants and if the bacteria overgrown also on the cotyledons, rinse with sterile water, dry for a few seconds on sterile paper, and transfer on B5/2 medium (0.8% agar) plus vitamins, plus cefotaxime, without sucrose.

5. Three days after washing the plants and removing the bacteria with cefotaxime, substitute parafilm with surgical tape and place the plants vertically to increase root growth. Remove any roots that do not emerge from the wound/infection site.

3.16 Screening of insertion mutagenesis lines LORE1

LORE1 transposition occurs in the germline, and harvesting seeds from a single founder line and cultivating progeny generates a complete mutant population. (Urbanski et al. 2012) To do so, one must select homozygous seeds from the original founder. This is done by performing a PCR screeing with positive and a negative reaction controls. The positive control is amplified with a primer designed in the gene region upstream the insertion and a second one designed in the LORE1 retro-transposon sequence; this confirms that there is an insertion in the expected locus. The negative control is amplified with primers designed in the 5' upstream region the insertion and 3' downstream, flanking the insertion site; amplification confirms a presence of at least one wild type allele and can clear which of the positive seedlings are heterozygous and homozygous for a given insertion event.

3.17 Vitro-vivo transfer of *L. japonicus* plants

Once *in vitro* plants achieve a fully developed root system (around 4-5 weeks post germination), they are transferred to soil. The soil is prepared with a mix of a part of sand, one part peat and two parts of soil (1:1:2) and sterilized in an autoclave. Plant roots are delicately cleared of agar deposition and transferred to soil and covered in pots covered with a wet transparent film cover. This step is required to allow a protective environment around the plant, providing it with the higher humidity environment required during the period of acclimatization to the new conditions. After one week, the film is removed and plants are watered every day. The growth of plants occurs in a growth chamber 23ºC and a photoperiod of 16h/8h and a luminous intensity of 246 µE s-1 m-2.

3.18 Collection and storing of seeds and pods of *L. japonicus*

Mature pods are collected and stored at 30ºC over one week, after which they are opened, seeds picked clean and organized for long-term conservation at 4ºC under darkness.

3. 19 Extraction of plant nucleic acids

3.19.1 Extraction of DNA from vegetal tissue

Around 0.2g of vegetal tissue is collected and immediately frozen at -80ºC. Samples are homogenized with a mechanic procedure through tissue lyser (QIAGEN) and to each sample frozen in the 2 ml eppendorf f 0.5 mL of Tris-HCl 50 mM (pH 8), EDTA 10 mM (pH 8), NaCl 100 mM, sarcosyl 1% and Ureia 10 mM solution is added. The mixture obtained is incubated for 37ºC for one hour, in constant shaking. At this point an extraction with Phenol:Chloroform:Isoamyl Alcohol 24:24:1 is performed, once again subjecting the sample to constant shaking. The samples are centrifuged at 7000x g for 5 minutes and the supernatant phase immediately recovered. The DNA is precipitated through addition of one volume of isopropanol and sodium acetate at a final concentration of 0,3M; this is followed by a centrifugation at 7000x g for 5 minutes and washing of DNA with ethanol 70%. After the DNA is dried, it is resuspended in TE buffer or water containing RNAse A (1µg/ml).

3.19.2 Extraction of RNA from vegetal tissue

The *Lotus japonicus* RNA extraction is carried out using a homemade protocol (Modified Kistner and Matamoros (2005) protocol). 20 to 30 mg of plant tissue are separated into two parts (shoot from root) in different 2mL eppendorfs and maintained briefly in liquid/solid nitrogen. Then, samples are either stored at -80ºC or immediately squeezed by tissue lyser (5-8 min at 30 Htz) into 550 µL of pre-warmed (65ºC) RNA extraction buffer (CTAB 2%; PVP 2% (MW 360,000); Tris-HCl 100 mM; EDTA 25 mM ; NaCl 2M; to be autoclaved) supplemented with DEPC 0,01% and β-Mercaptoethanol 2%. After a quick spin into micro-centrifuge, 550 µL of Phenol-chloroform-isoamyl-alcohol are added. The samples are vortexed and centrifuged (13.000 rpm) in the cold room for 10min. The supernatant is carefully recovered and the Phenol-chloroform-isoamyl-alcohol extraction is repeated a second time. The supernatant is recovered and transferred into new eppendorfs containing v/v (~450 µL) isopropanol. A gentle mixing of the extracted RNA is performed at room temperature time to time during 10 min. A final centrifugation in cold room is performed (13.000 rpm) to precipitate the RNA. The pellet is, subsequently, washed with 100-200 µL Ethanol 15% and re-centrifuged 5 min at max speed. The samples were dried by

vacuum manifold or safely under office light for 15 min. Pellets is dissolved in RNAse free water (DEPC water) in a volume that allows its total dissolution at an appropriate concentration for further manipulations. Samples are stored for a short period in -20ºC or at -80ºC for long period. The RNA is quantified by NanoDrop spectrophotometer (ND-1000). Just a single drop of 1.5 µL taken directly from the samples is loaded on the receptacle laser cell to provide an information on the concentration (ηg/µL) as well as the absorbance ratio 260/230 and 280/260 that must be around 2.0. The RNA quality check is performed by an electrophoresis running of 300 ηg RNA sample on agarose 2% Ethidium bromide pre-stained gel.

3.20 Electrophoresis migration of nucleic acids in agarose gel

Electrophoresis allows the separation of nucleic acid fragments according to their molecular weight and conformation. For the migration of DNA and PCR products of small dimension a 1.5% agarose gel is used, while for DNA of larger size the gel concentration was lowered to allow a better resolution of the gel bands. The agarose gel is prepared by solubilizing powdered agarose in TAE (Tris Acetate EDTA) buffer 1x; before pouring the gel in the electrophoresis tray, the intercalating agent ethidium bromide is added at the concentration of 0.33 µg/ml. The electrophoresis course is performed at room temperature at a constant voltage of 10 Volt/cm, using TAE 1x as course buffer. Analyzed samples are loaded in a solution of TAE 1X, 0.25% cylene cyanol, 0.25% bromophenol blue and 0.5% glycerol. An adequate marker is loaded alongside the samples, either 1Kb DNA ladder and/or 100 bp DNA ladder (Qiagen).

3.21 cDNA synthesis from RNA

In the semi quantitative and/or Real time experiment, cDNAs are synthesized by the support of the QuantiTect Reverse Transcription (QIAGEN Kit). Around 0.5µg of RNA is generally used from each sample extracted.

3.22 Polymerase Chain Reaction

Through PCR it is possible to amplify a specific DNA region, using two oligonucleotides (primer), complementary to the region immediately adjacent to our target DNA region. Around 100 ng of the DNA template is resuspended in a solution of 2.5 mM

MgCl2, PCR buffer 1x (original is 10x and contains 500 mM KCl, 10 mM Tris-HCl pH 9 e 1 % triton 100X) and 0.2 mM dNTP. To this mix are added oligonucleotides in a concentration around 30 mM. While there are many variations possible to the PCR program, the following is its basic structure:

Table 4: Standard PCR conditions

Step	Temperature (ºC)	Time	
Denaturation	94	5 min	
Denaturation	94	30 s	x35 cycles
Annealing	60	30 s	
Polymeralization	72	40 s	
Polymeralization	72	5 min	

The standard program often has an adjusted annealing temperature, depending on the melting temperature of the oligonucleotides, and elongation is modified depending on the size of the fragment that one desires to amplify (the polymerization step is usually 30 seconds per each 500 bp of the desired fragment). The PCR reaction is catalyzed by the termostable enzyme Taq polymerase.

3.23 Semi-quantitative PCR from cDNA library

RNA is extracted quality checked and quantified as described previously. Semi quantitative PCRs is performed taking as internal standard the LjUbiquitin gene (housekeeping gene). Gene amplifications are carried in 25 µl final volume of 0.25 mM dNTP, 2.5 mM $MgCl_2$ (Euroclone), Buffer 1x (Euroclone), Taq 0.2 U (Euroclone). All the PCR products are run on 1.5% agarose gel electrophoresis and Typhoon scanning gels was performed.

3.24 Root and nodule phenotypical analysis

All root and nodule phenotype analyses are performed in vitro. Final nodule numbers are accounted at 4 weeks post inoculation (but the kinetic of nodule formation is followed), while DNA and RNA recovery is performed both at 2 weeks (young nodules) and 3 weeks (mature nodules). Root kinetics and growth are checked every three days over the course of

two weeks, starting from seedlings in comparable stages of development. Two weeks old plants are sacrificed, their roots collected, dried over 48 hours in an incubator at 60ºC and their dry weight analyzed.

3.25 Shoot phenotype analysis

Shoot analysis is performed on two week old plants. After being sacrificed, shoot length and fresh shoot weight is measured. Shoots are dried 48 hour in an incubator at 60ºs degrees and then point dry shoot weight is measured.

3.26 Chlorate-sensitivity assay

Ever since AtNRT1.1 was found to be responsible for chlorate-sensitivity, this phenotype has been studied in various putative nitrate transporters. 4-day seedlings are germinated in *vitro* and transferred to vermiculite, subjected to N-deprived B&D medium, and treated with gradient concentrations of $KClO_3$ concentrations, using KNO_3 for a positive control comparison. Shoot development and leaf coloration are analyzed over the course of two weeks.

3.27 Uptake Assay in *Xenopus laevis* Oocytes

3.27.1 pGEM construct design

The entire coding sequence for our intended protein was amplified through PCR and inserted into a modified pGEM vector, pGEM-Xho. cRNA was transcripted through use of T7 Ultra kit and according to Ambion protocol.

3.27.2 Oocytes isolation

Females are anesthetized with benzocaine (~5ml 6% benzocaine in 100% ethanol) into 1L water, until the animal is limp and completely unresponsive. Ovaries are removed and washed with calcium and magnesium-free Ringers solution (110mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$m 2mM $NaHCO_3$, 5mM HEPES pH 7.8). Oocytes were dissociated with overnight treatment overnight at 16ºC in 0.1% collagenase in 5mg/mL ovalbumin in Ringers supplemented with 10 mM $NaPO_4$. Individual oocytes are recovered and are twice

washed in OR2 (82.5mM NaCl, 2.5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 1mM Na_2HPO_4, 5mM HEPES pH 7.8), supplemented with gentamycin (50µg/mL).

3.27.3 Oocytes selection

Using a dissecting microscope, mature stage VI oocytes for microinjection were selected. These oocytes are large, with good contrast between the black animal hemisphere and the creamy-colored vegetal hemisphere.

3.27.4 Oocytes microinjection

Oocytes were isolated and injected with cRNA codifying for your desired protein. Fill a previously calibrated injection needle (obtained by pulling a 6.6-µl micropipette Drummond with the Inject+Matic Puller) with the solution to be injected. For this, the microinjector is set on aspiration mode. To calibrate the injection needle make dot marks on the needle every 0.5 mm, which correspond to a volume of 50 nl. Insert the tip of the needle into an oocyte in the vegetal hemisphere, very close to the animal hemisphere, at an approximately 45-degree angle. Turn the microinjector setting to microinject, and microinject each oocyte with 50 nl of import substrate. The dot marks on the needle are used to monitor the amount of substrate that has been injected. Injected oocytes were transferred to a small (35mm diameter) Petri dish filled with OR2, and allowed to incubate for 48 hours at 20ºC, allowing them to express the intended proteins.

3.27.5 Nitrate uptake

After two days of incubation, oocytes are incubated for 90 min in ND96 solution (93.5 mM NaCl, 2mM KCl, 1.8 mM $CaCl_2.2H_20$, 2 mM $MgCl_2(.6H_2O)$, 5 mM HEPES pH 7.8) of different pH ranges and $^{15}NO_3^-$ concentration, dried for 24h at 80ºC and the amount of nitrate retained in the oocytes was determined through on a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (ANCA-GSL MS; PDZ Europa).

4. Results

4.1 Sequence analysis, discussion of the NRT1 and NRT2 landmarks

Lotus japonicus sequences with significant degrees of homology with genes codifying members of the NPF and NRT2 families were identified through in silico analysis. The genome of the model legume *Lotus japonicus* has been sequenced at 67% (472 Mb), covering around 91% of the genic space (19.848 coding sequences) (Sato et al. 2008) and the sequencing data are available in a public database at the website "Miyakogusa.jp" (as of April, 2014 at http://www.kazusa.or.jp/lotus. The BLAST analysis was reiterated also versus the data base of the expressed sequence tag (EST) of *L. japonicus*. The BLAST search was performed through the use of keywords in the search engine and an amino acids homology analysis of recovery sequences, comparing them with NPF and NRT2 proteins of *Arabidopsis thaliana*. This led to the retrieval of 38 *L. japonicus* complete NPF sequences most of which carry the typical 12 trans-membrane domains connected by short peptide loops. A further search led to the identification of 33 un-completed unique sequences of predicted *NPF* genes, indicating a size of around 70 members for the *L. japonicus* family that is consistent with the sizes of the *A. thaliana* and rice NPF families of 53 and 80 members, respectively. Fifty-one out of the 71 genes are physically mapped on the Lotus genome indicating a distribution on all the six chromosomes. The sequence distribution is not uniform over the 6 chromosomes of *L. japonicus,* as most are found on chromosomes 1, 2 and 4 with sixteen, fifteen and fifteen genes, respectively, whereas one gene is located on chromosome 3 and two on chromosomes 5 and 6. As expected, in many cases clusters were identified that possessed a high level of homology at nucleotide level (paralogous), originated from genic duplication events. These clusters are particularly numerous over the chromosome 2 and 3. This phenomena has been reported in *A. thaliana*, where numerous paralogues have been identified with a level of nucleotide identity even superior to 90%, found in all sorts of configurations (head-head, head-tail, tail-tail), that still manage to display independent regulatory elements and expression profiles. In fact, in *A. thaliana*, the elevated degree of sequence conservation associated to the grouping in clusters of 53 NPF genes, does not corresponds to a conservation of the spatial expression profiles or regulation in response to external stimuli, to the degree in which out of 51 expressed NPFs, only three gene copies

have perfectly overlapping expression profiles; this suggests a great diversity of functions for all family members that might be justified if one considers the amount of different local and dispersed patch of nutrient availability, how these can largely change in the environment and how the plants must respond efficiently and quickly to these. In our *in silico* search output, at least seven gene clusters were identified that co-localize to the same contigs with paralogous genes and short intergenic regions (e.g. 2032 bp between *LjNPF5.3* and *LjNPF5.4* genes ; Criscuolo et al. 2012). The criteria for the assignment of the 38 *L. japonicus* complete members to the eight clades of the NPF superfamily identified by Leran et al. (2014) and their relative position within these have been previously described in the Introduction section.

The BLAST search for *L. japonicus* NRT2 members was conducted with the same tools and criteria leading to the identification of four members.

A typical example of extremely conserved paralogues sequences is represented by two NRT2 orthologues in the chromosome 3, identified as the genes LjCM0649.40 and LjCM0649.30. They display very large homology with the *Arabidopsis thaliana* proteins NRT2.1 and NRT2.2 (79% and 78%, respectively). Alignment of the two Lotus paralogues with the program CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2) has revealed an almost complete nucleotide identity, with only 74 mismatches. The two sequences are in a tail-tail configuration and are separated by an intervening sequence of 3839 base pairs. This structural organization is perfectly identical to that of AtNRT2.1 and AtNRT2.2 and this is why we identified the *L. japonicus* genes as LjNRT2.1 (LjCM0649.40) and LjNRT2.2 (LjCM0649.30). Their regulatory sequence at the 5' does not share significant level of homology.

4.2 CM0608.1210.r2.m (LjNPF4.1) profile of expression in response to nitrate

When performing a screening of the expression of putative *L. japonicus* nitrate transporters orthologues to Arabidopsis NPFs and NRT2 genes upon root and nodule tissues, Criscuolo et al. (2012) found that the expression of the chr2.CM0608.1210.r2.m (*LjNPF4.1*) gene was induced in roots after shifting the 2 weeks old plants from a condition where the only N source was 1 mM glutamine to increasing concentrations of KNO_3 as the sole N

source, in a range of concentrations from 0.01 mM to 2 mM for a time-course experiment extended up to 48 hrs from the transfer. *LjNPF4.1* was the only gene, among the 9 characterized NPF members, to be induced in both high and low nitrate conditions. This result was consistent with literature data where only a few NPF genes are reported to be induced by nitrate. In addition to the nitrate-induced expression profile in root we reported in Criscuolo et al. 2012, we later analyzed the expression profile of *LjNPF4.1* in the shoot of plants subjected to the same conditions in which we found nitrate-induction in roots, in a range of concentrations from 0.01 mM to 2 mM for at 10 hrs and 48 hrs after inoculation (Fig. 17). In a stark contrast with the clear cut induction by nitrate in roots, we observed no nitrate-dependent induction of LjNPF in shoot tissue.

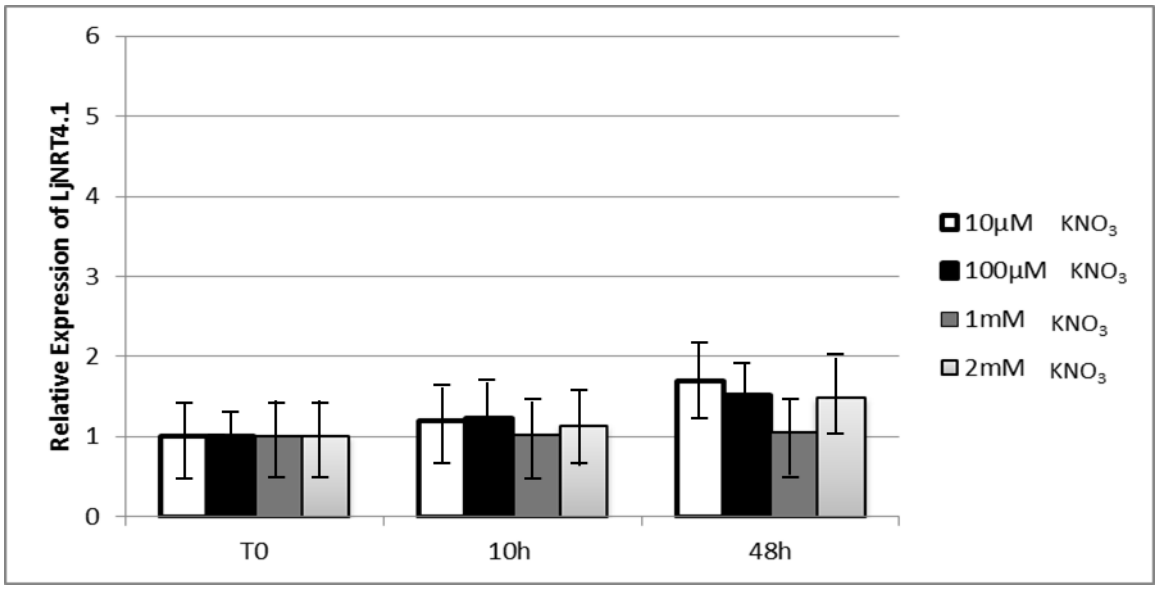


Fig. 17: Time-course analysis of relative expression of LjNRT4.1 in leaf tissue in response to difference concentrations of nitrate. Leaves samples were collected 10 or 48 after transfer from 1mM Gln condition to 10 µM, 100 µM, 1mM or 2mM $KN0_3$ and their LjRNT4.1 transcripts analyzed through PCR. Untreated plants were used as control.

Such an organ-dependent profile of nitrate induction has been reported also for *A. thaliana* NPF members (AtNRT1.2; Okamoto et al. 2003). However, LjNPF4.1 stood out amongst the nitrate-induced NPF that we reported because it was the only one out of the nine analyzed Lotus NPF to show an amount of root transcript increased by both nitrate conditions. The peak of induction was observed very quickly at 10 hrs after the shift (about 4

fold) and although reduced later on, a significant level of induction (about 2 fold) was maintained up to 48 hrs after the shift from glutamine to KNO_3 (Criscuolo et al. 2012). This dual profile of induction lent towards the hypothesis that NPF4.1 might have a dual-affinity transporter activity, being able to uptake nitrate in both poor and abundant environmental availability. So far, only two NPFs with this capacity have been reported: Arabidopsis NRT1.1 (NPF6.3) and Medicago NRT1.3 (NPF6.8) (Liu et al. 1999; Tsay et al. 2007; Morere-Le Paven et al. 2011). However, for our aims the peculiar pattern of response to nitrate was of particular interest as this might be the expected profile of a transporter gene involved in the nitrate-dependent response of the nodulation pathway.

4.2 GUS spatial profile of expression compatible with a role in nitrate uptake

In order to obtain crucial information needed to explore the biological role played by LjNRT4.1 and its putative link with the control of nodule organogenesis processes, we decided to analyze the spatial profile of expression of this gene in *L. japonicus* roots and nodules.

This approach was followed by making a T-DNA construction carrying the regulatory region of the LjNRT4.1 gene fused to the *gusA* marker gene. The promoter region was retrieved through *in silico* analysis of the *Lotus japonicus* genome database. In particular, a fragment of 716 bp including 689 bp upstream of the start codon of LjNRT4.1 and 30 bp downstream of the ATG (coding for the first ten amino acids) was sub-cloned in the plasmid vector containing the promoter-less reporter gene *gus*A (pBI101.1 vector), for obtaining the translational fusion construct promLjNPF4.1-*gus*A. The sub-cloning strategy was the following: the forward and reverse primers designed for the amplification of the 5' region included the recognition sites of the *Sal*I and *Bam*HI restriction enzymes, respectively (Table 5 and 6). The amplification product (Table 5 and 6) was first ligated into the pCR2.1-TA cloning plasmid (Invitrogen; Fig. 18), an appropriate linearized vector carrying T residues at the 3'-ends for facilitating the sub-cloning of Taq polymerase PCR-amplified fragments with A-overhangs. This preliminary step was necessary because the amplified 716 bp fragment contains an additional *Bam*HI site 196 bp upstream of the ATG start codon, hence requiring a

partial *Bam*HI digestion for the next subcloning step in the pBI101.1 T-DNA vector carrying the *gus*A marker (Fig. 19). The subcloning in the pCR2.1 plasmid allowed obtaining a high amount of DNA easier to be handled and hence the *Bam*HI partial *Sal*I complete digestion was performed to obtain the whole 716 fragment for ligation in the *Bam*HI-*Sal*I double digested pBI101.1 T-DNA vector. This ligation strategy allowed the sub-cloning of the first 10 codons of the LjNPF4.1 gene (at the 3' of the 716 bp amplified fragment) in frame with the ATG codon of the *β-glucuronidase* gene. The appropriate frame of the resulting plasmid was checked by sequencing, before electroporating the construct in the *A. rhizogenes* 15834 strain.

Table 5: Different oligonucleotides used for the subcloning of the 5' regulatory region of the LjNPF4.1 gene. The *Sal*I and *Bam*HI restriction recognition sequences are indicated in red and blue, respectively.

Name	Sequences (5' to 3')	T_m (ºC)	Amplicon size (bp)
Lj608.1210F2gus	GCGTCGACGACACTATCTAATTAGTAAT	68	716
Lj608.1210Rev-gus	GGGATCCTCTGGTAACTTGGCCTGCTTCT	67	

Table 6: PCR conditions for amplification of the LjNRT4.1 promoter region for the Gus fusion construct.

Step	Temperature (ºC)	Time	
***LjNRT4.1 Promoter Region* (Lj608.1210F2gus/ Lj608.1210Rev-gus)**			
Denaturation	94	5 min	
Denaturation	94	30 s	x35 cycles
Annealing	60	30 s	
Polymeralization	72	60 s	
Polymeralization	72	10 min	

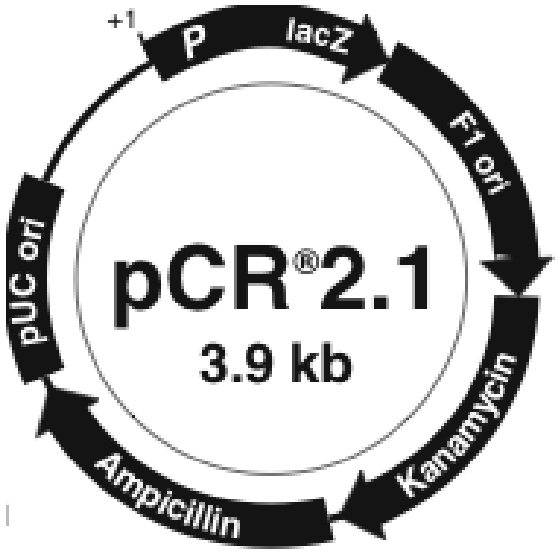


Fig. 18: Map of the pCR2.1-AT cloning. The polylinker (PL) for subcloning is located between the P and *lac*Z sequence. The restriction sites in the PL and the linearization site with 3' protruding ends are in the following order from left to right: *Hind*III, *Kpn*I, *Sac*I, *Bam*HI, *Spe*I, *Bst*XI, *Eco*RI 3'T - T-3' *Eco*RI, *Eco*RV, *Bst*XI, *Not*I *Xho*I, *Nsi*I, *Xba*I, *Apa*I.

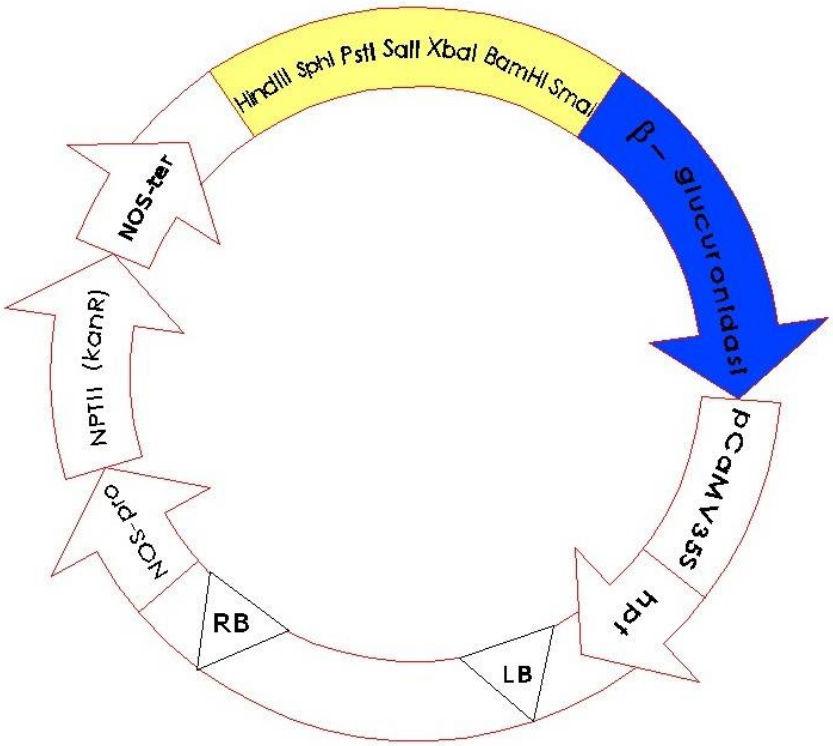


Fig. 19: Map of the 8.5 kb T-DNA vector pBI101.1. The restriction sites of the polylinker (yellow), *gus*A marker (blue), regulatory regions (white), hygromycin resistance cassette (hpt) and the Right and Left borders (RB and LB) are indicated.

For the spatial analysis of the LjNPF4.1 promoter activity we exploited the hairy roots methodology, which allows to obtain transgenic root tissues transformed with the appropriate construct that in our case is represented by the LjNPF4.1prom-*gus*A fusion. The transformed hairy root system is developed after infection with *Agrobacterium rhizogenes* 15834 strain. This represents a short-cut methodology that allowed us to obtain a composite plant (wild-type shoot and transgenic root system) to test the promoter activity, by avoiding the very long procedure of plant regeneration that must be exploited when resorting to an *A. tumefaciens*-mediated transformation.

Microscopic analyses of the GUS activity of roots transformed with the fusion construct LjNPF4.1prom-*gus*A (Fig. 20) reveals a strong promoter activity of NPF4.1 across the root system. This root expression is especially pronounced on the root epidermis and the cap of the root in the primary and secondary roots tips (Fig. 20 A-E), with occasional vascular expression (Fig. 20 B, C). This pattern was also confirmed in 60 µM root cross sections obtained at the vibratome (Fig. 20 F). The pattern of GUS activity was followed in hairy roots of plants grown in different N regimes and after shift from glutamine 1 mM or no Nitrogen media to different KNO_3 concentrations (low 100 µM or high 2 mM) to identify possible changes correlated to different growth conditions. The overall analysis did not reveal any qualitative change in terms of spatial distribution of the promoter activity that was maintained in all the tested conditions. In addition to these findings the expression of the *LjNPF4.1* is not extended to any nodular tissue, being specific to roots (data not shown). This type of GUS distribution is consistent with that expected for a transporter involved in nitrate uptake from the external medium. A similar role has already been reported for the *A. thaliana* AtNPF6.3 (old name NRT1.1) and AtNPF4.6 (old name AtNRT1.2) genes. Interestingly, as shown in Table 2, the latter *A. thaliana* gene shares the same sub-clade 4 classification as LjNPF4.1 (Léran et al. 2014).

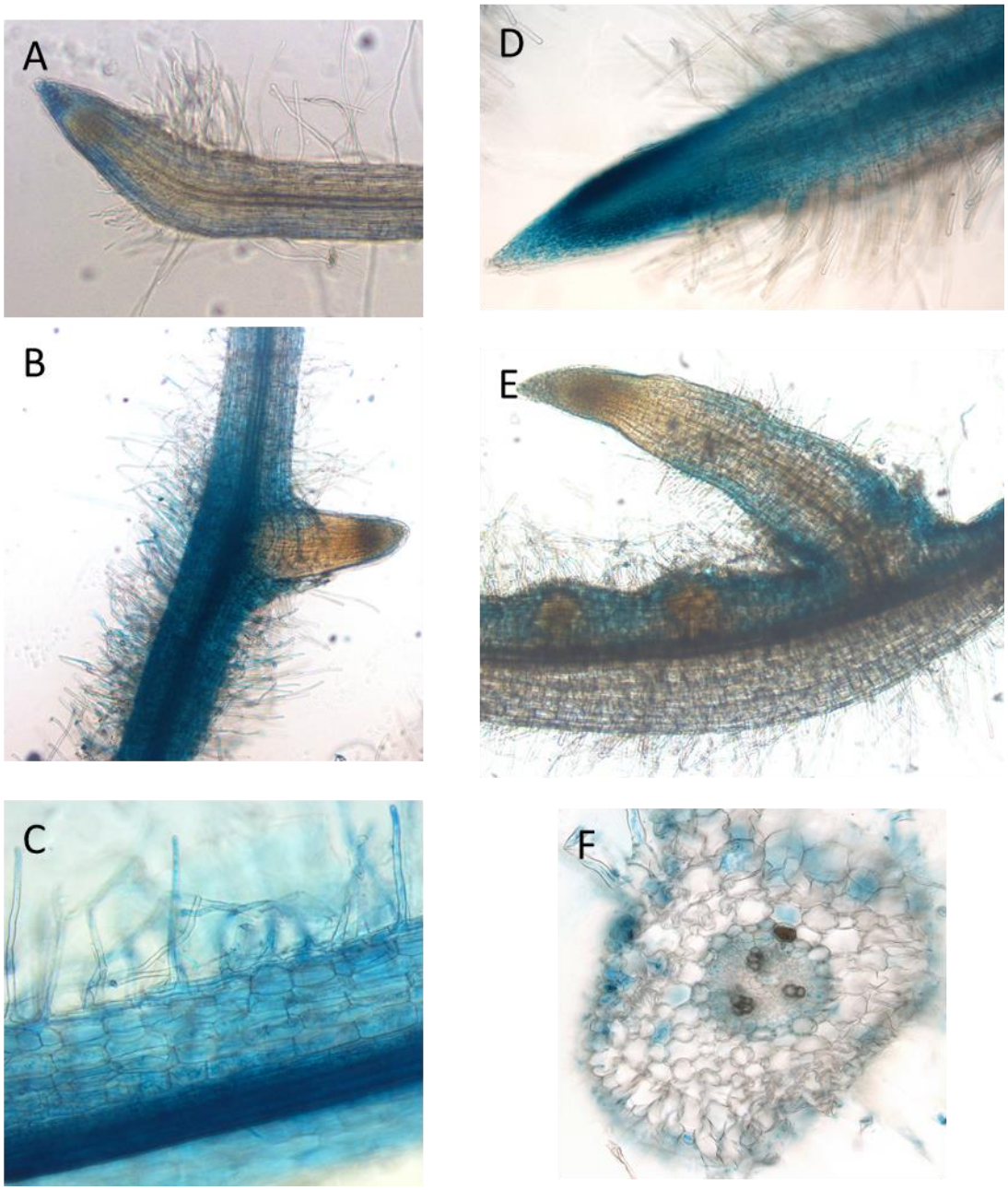


Fig. 20: Analysis of the spatial profile of the fusion construct LjNPF4.1-pr-*gus*A in *Lotus japonicus* hairy roots. Roots incubated 24h with: A) 100 µM KNO_3; B) N-Free Root; C-E) 2 mM KNO_3; F) 60 µM cross root section of root.

The sequence of 5' 689 bp region upstream of the coding LjNRT4.1 region that drives the root tissue pattern of expression of *gus*A is shown in Fig. 21. This was analyzed for the presence of *cis*-specific motifs associated with the response to nitrate (Nitrate Responsive Elements, NRE). There are different types of DNA NRE motifs described in literature, although they are randomly represented throughout various plant genomes, and many times a strict relationship of the reported consensus sequences with the transcriptional

response to the presence of nitrate was not confirmed experimentally. We focused our search for the two most prevalent NRE motifs: Ag/cTCA and GATA. Ag/cTCA core sequence motif is normally preceded by a 7-bp AT rich region found in genes involved in nitrate assimilation (Das et al. 2007). We found two Ag/cTCA sequence represented in the 689 bp 5' region of LjNPF4.1 and only one was preceded by an AT rich region (Fig. 21). In the case of GATA box, this is the target binding site of the GATA transcription factors broadly distributed in eukaryotes (Reyes et al. 2004). These are implicated either in the light-dependent and nitrate-dependent control of transcription in plants and we can find 5 putative GATA binding sites in the LjNPF4.1 5' regulatory region (Fig. 21).

GACACTATCTAATTAGTAATTAGAGTATGAGAGAAAAATTATA**GATA**TATATAAAATGAT
GAAGTGATATTTGTTAGAGTTGTCACTTTTTAATTTCGAAAATAGTATATAATATAA**GATA**T
ATTCTACAACCATAT**GATA**AGAATTACAACTGTTGATTTATCCAATAATTTTCAT**GATA**ATT
TAGTTTATCATTTAAATTTATGGTTCTCTCACCATAAAGAAAATTAATAATTAATGTAACTA
ATTAAAAAATATATAAGAAAATAATATATACACTGACATCATACAAAATGTTAAAAGATCAT
ATTATATAGTAAAATAATAATATTATAGTTGTAATATATTTTATCGTTATTTTAACATATTT
TGGACCGAACAA**GATA**CCATGTCTTAAATGTGAAGAGAAAAAA*AGTCA*ATGTGATGGTTCAT
GAATGGCGTTGTATCTTGTGATGACCATTCTGCCCAAGCAGGAGCCATCCTTGCTGTACCT**GG**
ATCCTCTTTAAATCTCTCACC*ACTCA*TCTTTCTTTTCTCCCTCAACCCCTTCTCAGAATCCAAA
GCCAATAACGTGGGTTCTCTCTCTCTCTATCTCTCTCCTCTCCCCTTTTCTATTGATTACTTTC
ACAAAGTACTATATAGCTAGTAACTAATATTTTCACTAATTATACAATTGTATCTATCTGCA
G**ATG**GAACT**AGAAGCAGGCCAAGTTACCAGA**

Fig. 21: Regulatory 5' region of the LjNPF4.1 coding region. The primers used for promoter-GUS analysis are indicated in orange, the start codon is in red and the *Bam*HI site is in green. The putative Ag/cTCA regulatory motifs are underlined in italic font. The putative GATA regulatory motifs are in bold font.

However, the biological relevance of these motifs could be only tested by a deletion analysis of the 5' region and/or DNA binding experiments that represent a matter out of the purpose of this thesis.

The significance of the observed profile of spatial activity of the LjNPF4.1 gene is further underlined by the comparison with the pattern of promoter activity of other LjNPF genes. In fact, our ultimate aim is to elaborate a complete atlas of spatial gene expressions

for the LjNPF and LjNRT2 families, which will provide crucial information for the understanding of the roles played by these proteins in different plant processes. As an example, I describe here the profile of expression of the LjNPF2.2 (*Lotus database* name CM0608.1290). Even in this case I obtained a translational fusion between the 1071 bp of the LjNPF2.2 5' regulatory region (including the first 10 codons of the gene) to the ATG of the *gus*A marker through a *Sal*I- *Bam*HI ligation in the pBI101.1 T-DNA vector. After elettroporation in the A. rhizogenes strain 15834 and transformation of Lotus plants, the pattern of GUS activity observed in hairy roots was completely different from that observed with the prLjNPF4.1-*gus*A. The blue staining is confined to the root vascular structures and no promoter activity is detected in the root tip and epidermal zones (Figure 22 A, B). The cross section in Fig. 22C confirms the root vascular stele specific profile of expression, also indicating stronger staining into the xilematic vessels, suggesting a function in the unloading of nitrate. Such a role has been proposed for the AtNRT1.8 gene, which shares a similar spatial distribution of promoter activity with LjNPF2.2 (Li et al. 2010).

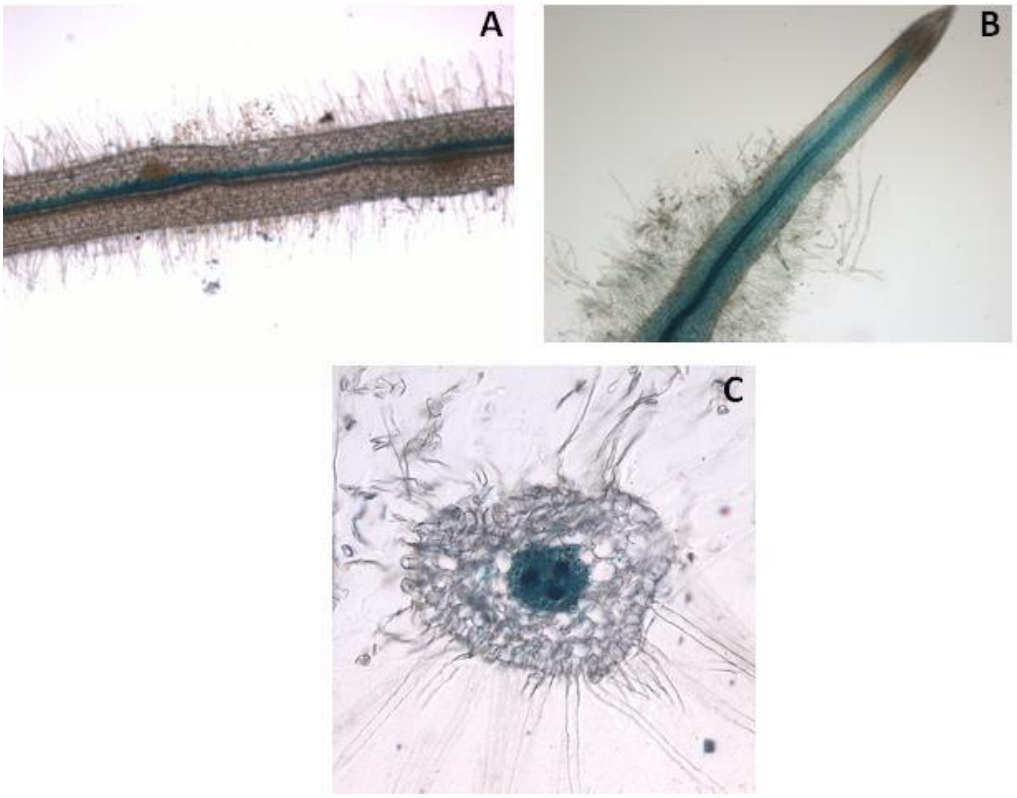


Fig. 22: Representative pattern of GUS activity in transgenic hairy roots transformed with the LjNPF2.2-prom-*gus*A fusion. A-B) Staining in whole root; C) 60 µM cross section of root.

4.3 Biochemical characterization of *LjNPF4.1*

4.3.1 Nitrate uptake by *LjNPF4.1*

While LjNPF4.1 shows a root nitrate-dependent induction and a spatial profile of expression fitting to that expected for a nitrate transporter, the actual capability for this protein to import nitrate or other putative substrates must be assessed. We exploited the *Xenopus laevis* oocytes system to study the transport activity of various Lotus NPF family members; *Xenopus* oocytes offer a heterologous expression system, able to efficiently transcribe and translate heterologous genetic information and perform the assembly of the foreign protein product after being microinjected with cRNA coding for such protein.

The coding sequence of LjNPF4.1 (Fig. 23) was amplified through high fidelity PCR reaction, and inserted in a *Xenopus* oocytes transcription and expression vector. The vector used was pGEM-Xho, a version of pGEM-He modified at the University of Erlangen-

Nuremberg with the deletion of an ATG sequence from the polylinker; this vector contains 3' and 5' untranslated regions (UTRs) of a *Xenopus* β-globin gene (Fig. 24).

TCATGAATGGCGTTGTATCTTGTGATGACCATTCTGCCCAAGCAGGAGCCATCCTTGCTGTACCT
GGATCCTCTTTAAATCTCTCACCACTCATCTTTCTTTTCTCCCTCAACCCCTTCTCAGAATCCAA
AGCCAATAACATGGAACTAGAAGCAGGCCAAGTTACCAG**ATG**GGAAGGCTATGTTGATTGGAG
GAGCAGGCCTGCTCTTAGAGGCAGCCATGGAGGCATGCTTGCAGCCTCCTTCGTTCTGGGTGTG
GAGATTTTGGAAAATTTGGCGTTTTTGGCCAATGCCAGCAATTTGGTATTGTACTTGAAGCAGT
ACATGCACATGTCACCTTCGAAATCTGCCAATAATGTCACTAATTTCATGGGAACCGCCTTCCTC
CTTGCACTTCTTGGTGGTTTCTTATCCGATGCATTTTTCACTTCTTATCATGTCTACCTGATAAG
TGCACTTATTGAGTTCCTGGGTTTGATTGTACTCACCATACAAGCTCGTTCACCTTCACTAAAGC
CACCACAATGTGATGAAGGCACCATATGTCAGGAAGTTAATGGTGGAAAAGCAGCAATGTTGTT
TGCTGGCCTCTATCTGGTGGCTCTTGGAGTTGGAGGAATCAAAGGATCATTGCCAGCACATGGT
GGTGAGCAGTTTGATGAAAGCACCCCAACTGGAAGAAAGCAGAGATCAACCTTCTTCAACTAC
TTTGTGTTCTGCCTATCATGTGGTGCCCTTATTGCTGTTACTCTTGTGGTGTGGGTTGAAGACA
ACAAAGGATGGGAATGGGGTTTTGCAATATCTACAATTACCATATTTGTATCCATCCCATTGTTC
TTGGCAGGCTCTACTACTTACAGGAACAAGATCCCTTCAGGAAGCCCCCTCACAACCATTTCAA
AGGTTCTTATTGCTGCTATACTGAATTGCTGCTGCACCAATAAAAACTCTAGCAATGCTGTTGT
GAATATGGTGTCAAGCCCTTCTGATCCACACTCAGGTAGAAAAGAATCAGTGGAAGAAACTAAC
AAAGCAAGCACATCAGCTGAAACCCCATCAGAGTCCCTCAAATTCCTTAATGGAGCAGCTGCAA
ACAAGCCAGTATTTTCGTCATTAGAATGCACTGTACAACAAGTTGAAGATGTCAAGATAGTATT
GAAGGTACTGCCTGTATTTGCCTGCACCATTATGCTGAACTGTTGCTTGGCTCAGTTGTCCACA
TTCTCTGTTGAACAAGCTGCTACAATGAACACCAAATTGGGTTCCCTCAAGGTGCCACCGGCTT
CTTTACCAGTTTTCCCAGTGCTCTTTATCATGATCCTAGCACCAATATATGACCATGTTATTATCC
CTTATGCTCGGAGAACGACGAAATCAGAAATGGGCATCAGTCATCTCCAAAGGATTGGAATTGG
ATTAGTACTCTCTATAGTTGCCATGGCTGTGGCTGCTGTTGTTGAAGTGAAAAGGAAAAGGGTG
GCCACTCACTCAGGCCTAGTTGATGATGCTACCAAACCACTACCTATCTCATTCCTTTGGATTGC
TTTTCAGTACTTATTCCTTGGCTCTGCTGATCTTTTCACCTTGGCTGGGTTGTTGGAGTTTTTC
TTCTCAGAAGCACCAATAAGGATGAGATCTTTGGCCACATCACTTTCATGGGCCTCTTTGGCAA
TCGGGTACTACCTAAGTTCAGCCATTGTATCAATAGTAAACAGTGTCACTGGTAAAGGCTCCCAC
AAACCATGGCTATCTGGTGCCAACCTTAACCACTATCACCTAGAGAGGTTCTATTGGCTCATGTG
TTTGCTGAGTGGGTTGAACTTCCTACATTACCTGTATTGGGCGGCTAGGTATAAATATAGAGGG
AGAGGTACTGCTAATGAGTGAAGCAATTGTGGGAGTTTTTCAAACAGTCTATATGTGCACCTT
AGTTTAAGGGTTCATTGGTCAAAGTATTTCCAAGCCATTCTTCAAAAGGGCGCATCAGCATTTT
CCTCCGTAGAGATAGTATAGAGATATCATGCACATACAAGAATAACA

Fig. 23: Coding region of LjNPF4.1. At orange, the primers used for amplification and construction of the pGEM-Xho vector, with the initiation and termination codons underlined.

Table 7: Sequences of oligonucleotides used for amplification of the LjNRT4.1 coding region. In bold are the sequences for the restriction enzymes required for ligation: blue is *BamHI* and red is *HindIII*.

Name	Sequences (5' to 3')	T_m (ºC)	Amplicon size (bp)
Lj608.1210XhLF	CG**GGATCC**TCTATCTGCATGGAACTAGAAG	60	1755
Lj608.1210XhLR	CC**AAGCTTA**CAATTGCTTCACTCATTAGCA	58	

Table 8: PCR conditions for high-fidelity PCR amplification of the LjNRT4.1 coding sequence for heterologous expression construct.

Step	Temperature (ºC)	Time	
***LjNRT4.1 High-Fidelity* (Lj608.1210XhLF/XhLR)**			
Denaturation	94	5 min	
Denaturation	94	15 s	x45 cycles
Annealing	52	60 s	
Polymeralization	72	110 s	
Polymeralization	72	10 min	

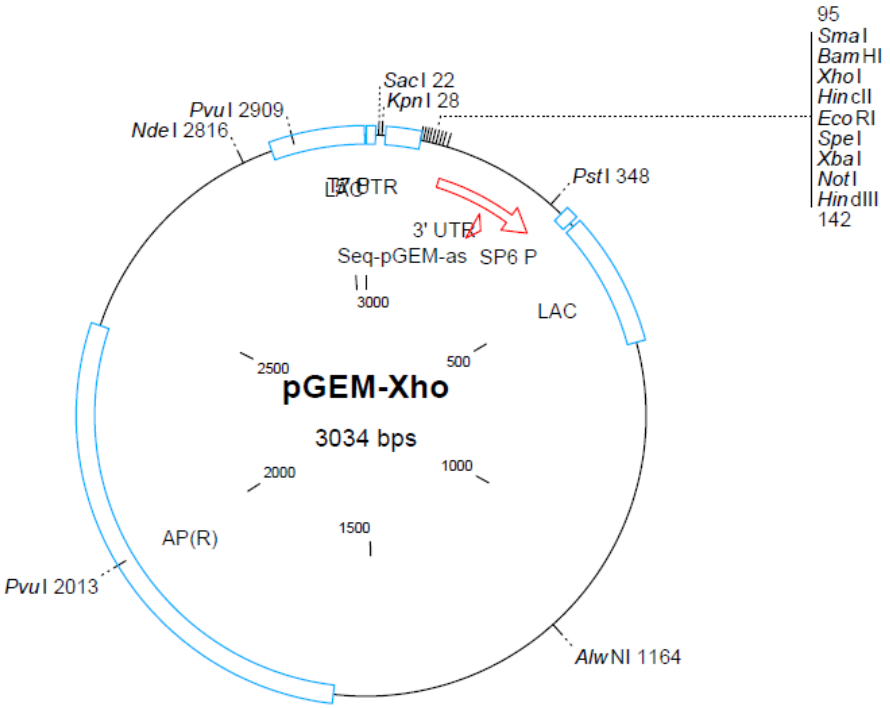


Fig. 24: Map for the heterologous expression pGem-Xho vector used for *Xenopus laevis* oocytes assays. The polylinker region is showcased between 95 and 142 bps. The vector contains lac operon ad T7 and Sp6 promoters, required for RNA transcription; ampicillin resistance gene is used for selection.

The primers used for amplification of LjNRT4.1 added to the sequence restrictions site for *BamHI* and *HindIII*. The pGEM-Xho vector was double-digested with *BamHI* and *HindIII*, successful digestion confirmed through gel electrophoresis and the fragmented recovered and purified. The LjNRT4.1 sequenced amplified through high-fidelity PCR (Table 6 and 7) was double digested with the restriction enzymes *BamHI* and *HindIII*. Assessed through gel electrophoresis, the digestion products were recovered and purified. The double-digested *BamHi/HindIII* sequence was ligated with double-digested *BamHI/HindIII*, the resulting fusion pGEM-Xho-LjNRT4.1 (Fig. 25). Construct was validated through nucleotide sequencing.

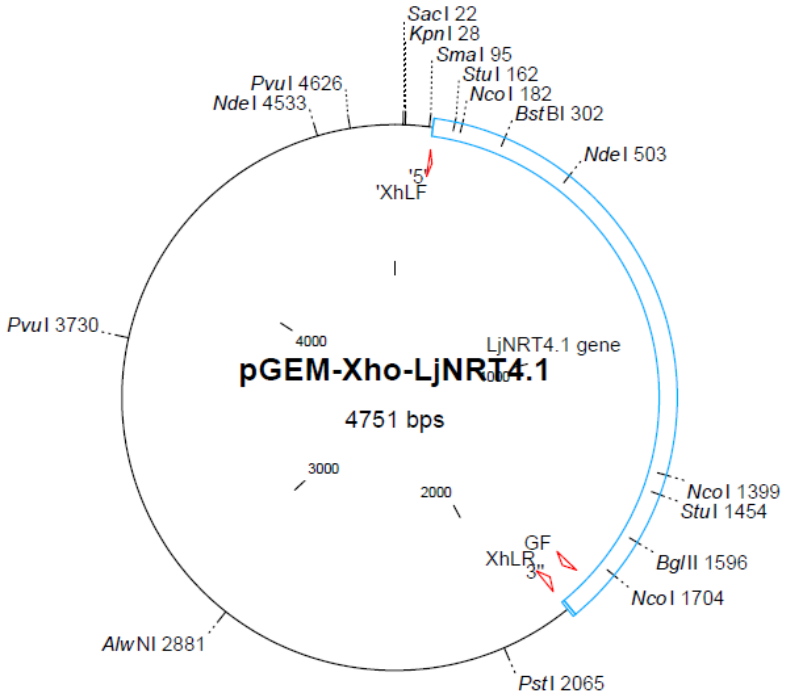


Fig. 25: Map for the pGem-Xho-LjNRT4.1 vector used for heterologous expression in *Xenopus Laevis* oocytes.

cRNA was transcripted from the linearized pGEM-Xho-LjNRT4.1 construct, and microinjected into *Xenopus* oocytes, which were then incubated for 48 hours at 20ºC. After such period had elapsed, *Xenopus* oocytes expressing LjNRT4.1 and control oocytes microinjected with water were subjected for 90 minutes to ND96 solution of pH 5.5, 6.5 and 7.5, and either 1mM or 30 mM of radio-labelled $^{15}NO_3^-$. This range of conditions allowed us check the nitrate affinity of the LjNPF4.1 protein and its possible pH-dependent transport activity.

LjNRT4.1 displays an unambiguous low-affinity transport activity, being able to uptake nitrate only in the presence of a high concentration (30 mM) and at a pH of 6.5, confirming that this uptake is proton-dependent (Fig. 26). However, although less pronounced, LjNRT4.1 also seems to have some level of high-affinity transport activity, being able to uptake nitrate even at low concentration (1 mM), again at pH 6.5. This very specific and narrow window of activity is particularly interesting; while it is evidence of a proton-dependent nitrate transport activity for LjNRT4.1, most of the reported low-affinity

transporters in other plants have some degree of uptake activity in acidic conditions, with an arrest of uptake occurring after a neutral or alkaline threshold. As mentioned above, Criscuolo et al. 2012 have previously reported an increase in LjNRT4.1 transcription of plants treated with 100 µM and 2mM nitrate, results that partially match these dual nitrate uptakes findings. The dual-affinity nitrate transport activity is also an unusual feature amongst NPF family members as AtNPF6.3 and MtNPF6.8 are the only two NPF members with a reported to proton-dependent low and high affinity nitrate transport activities. It is relevant to point out that while AtNPF6.3 and MtNPF6.8 are both categorized in the same sub-clade 6 of the NPF family, LjNRT4.1 is classified in the same clade as ABA/Ga3 transporter AtNPF4.1 and ABA/nitrate transporter AtNPF4.6 involved in the nitrate uptake (Table 2).

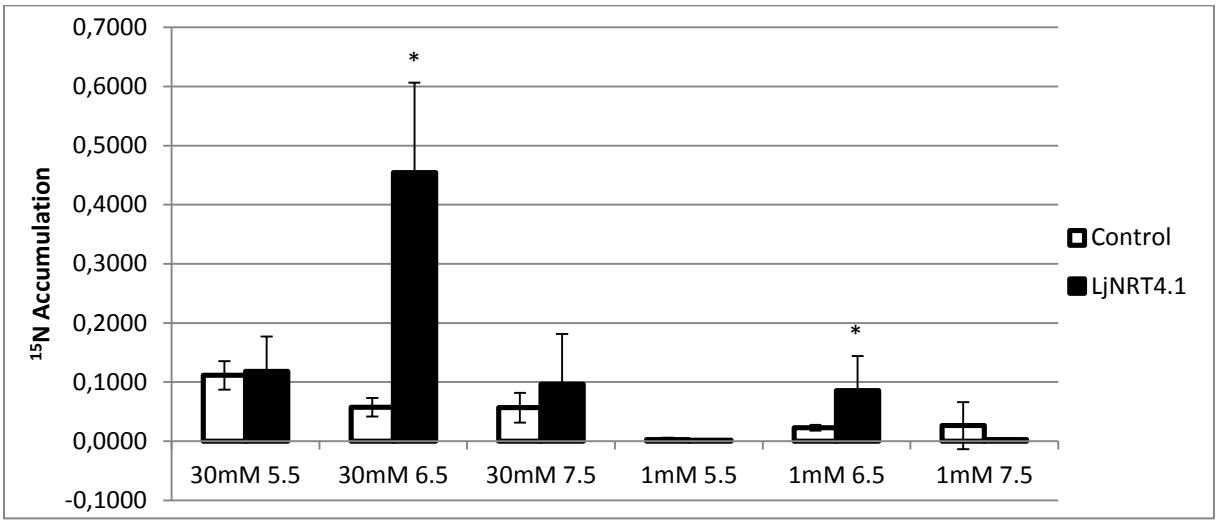


Fig. 26: ^{15}N accumulation inside of *Xenopus laevis* oocytes treated radio-labelled $^{15}NO_3^-$. Control (water injected) and LjNRT4.1 expressing oocytes were treated for 90 minutes at the same conditions of either 1 mM or 30 mM of $^{15}NO_3^-$ at the pH of 5.5, 6.5 or 7.5. Each data point is the mean ±SE for 8–10 oocytes. * p ≤0.05.

4.3.2 Phenotypical characterization of knock-out *Ljnpf4.1* plants

In order to complete the characterization of the LjNPF4.1 to investigate the role played by this transporter in the Lotus growth and developmental program, we followed a reverse genetic approach to obtain plant lines deficient in the expression of LjNPF4.1. Recently, a terrific tool has been set up by the Center for *Carbohydrate Recognition and*

Signalling (CARB) in Denmark: a very large collection of LORE1 – Lotus retrotransposon 1 – insertion mutagenesis lines has been obtained and characterized through the sequencing of the flanking regions of more than 40000 insertion events through the *FSTpoolit* protocol described in Urbański et al. 2011. This gene tagging tool characterized by the pollen specific stage of transposon jumping, is comparable to that obtained through floral-dip T-DNA transformation of Arabidopsis plant and its capacity to produce independent germinal insertions, thereby allowing generation of mutant populations from seeds of single plants; LORE1 offers a high-efficiency of insertions with a predilection for the exons of protein-coding genes and the aforementioned protocol offers a reliable, high sensitivity and specificity procedure to identify and catalogue those insertions, allowing the creation of this gene tagged database. This offers an alternative to the laborious tissue culture transformation protocols that are the only other alternative in order to generate stable T-DNA transformed lines.

These LOREI lines have been characterized through blasting of the sequences flanking every LORE1 insertions (1000 bp sequenced upstream and downstream of the insertion site) in order to obtain a list of L. japonicus genes that were tagged by the retrotransposon insertion event. The different lines carry a different number of LORE1 lines (from 1 to 12, average 5 insertion per line) that segregate through the generations. Therefore, a simple blast analysis of the LjNPF4.1 genomic sequences versus the bank of collected LORE1 flanking sequences allowed the identification of the LORE1 line N° 30000742 carrying 10 independent LORE1 elements, one of which is inserted into the third exon of the LjNPF4.1 gene (Fig. 27). However, the zygoty of the requested batch of first generation of seeds is unknown. As such, it is crucial to analyze the segregants of any specific LORE1 line, by an analysis on their genomic DNA and PCR screening for plants homozygous for insertion in the desire genes of interest.

Screening of successful insertion events and homozygosity was performed in two steps, as previously described in the Material and Methods section of this thesis:

a) Using oligonucleotides complementing the region upstream and downstream of the insertion event, it is possible to discern if there was disruption of the sequence in both alleles, a form of negative screening (Fig. 27). The

oligonucleotides 742Frw and 742Rev, complementary to the LjNRT4.1 sequence were utilized for this screening (Table 9 and Fig. 27) ;

b) Using oligonucleotides complementing the region upstream of the insertion and the LORE1 sequence, it is possible to confirm if there is indeed a successful insertion of the transposon in the target gene (Fig. 27) The oligonucleotides 742Frw, complementary to the LjNRT4.1 sequence, and P2, complementary to the LORE1 sequence, were utilized for this screening (Table 9 and Fig. 27) .

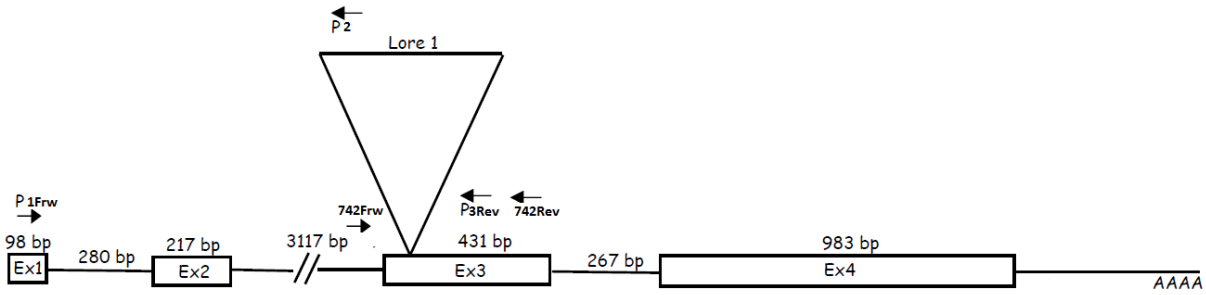


Fig. 27: Rough scheme of the relative positions of the oligonucleotides used to confirm successful LORE1 insertions in the LjNRT4.1 gene and assess expression levels.

Table 9: Different oligonucleotides used in LORE1 screening and LjNRT4.1 expression analysis.

Name	Sequences (5' to 3')	T_m (ºC)	Amplicon size (bp)
742Fwd	GCTCGTTCACCTTCACTAAAGCCACCA	63	405
742Rev	TGAAATGGTTGTGAGGGGGCTTCC	61	
742Fwd	GCTCGTTCACCTTCACTAAAGCCACCA	63	435
P2	CCATGGCGGTTCCGTGAATCTTAGG	62	
Lj608.1210.P1Frw	AGGCCAAGTTACCAGATGGG	63	592
Lj608.1210.P3Rev	AACGAGCAATAAGGGCACCAC	62	
LjUbiFrw	TTCACCTTGTGCTCCGTCTTC	64	90
LjUbiRev	AACAACAGCACACACAGACAATCC	70	

LORE1 seeds were sterilized and germinated on B5/2 medium. Leaf material was collected from one week old plants (~10 mg) and genomic DNA was extracted. This DNA was used as the template for the aforementioned screening PCR reactions. The conditions utilized in these reactions are presented in Table 10.

Table 10: PCR conditions for the different oligonucleotides.

Step	Temperature (ºC)	Time	
742 WT/742 P2			
Denaturation	94	5 min	
Denaturation	94	30 s	x35 cycles
Annealing	64	30 s	
Polymeralization	72	40 s	
Polymeralization	72	10 min	
P1F/P3R (Expression)			
Denaturation	94	5 min	
Denaturation	94	30 s	
Denaturation	94	30 s	x30 cycles
Annealing	60	30 s	
Polymeralization	72	40 s	
Polymeralization	72	10 min	
Ubiquitin			
Denaturation	94	3 min	
Denaturation	94	30 s	x23 cycles
Annealing	63	30 s	
Polymeralization	72	30 s	
Polymeralization	72	5 min	

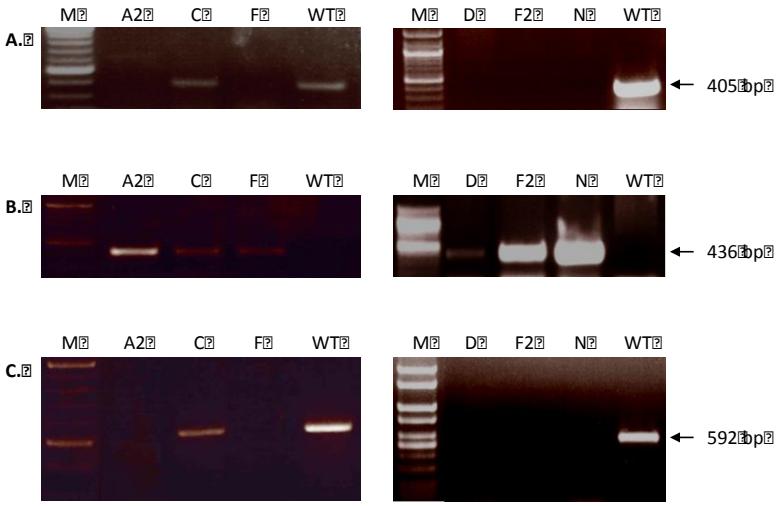


Fig. 28: PCR screening on segregants of the LORE1 30000742 line.

DNAs from plants of this line were screened for unmodified wild-type sequence in the LjNPF4.1 gene and for a successful insertion event in the region flanking the LORE1 insertion in the third exon of the gene (Fig. 28). The representative results of the PCR-based screening described above (Fig. 28) conducted on genomic DNAs extracted from six random segregant plants of the LORE1 line 742 are summarized in Fig. 28. As shown in Fig. 28A, five out of the six plants (A2, F, D, F2, N) do not amplify the expected wild type band of 405 bp (Table 9; 742Fwd + 742Rev) and all of these (Fig. 28B) showed the expected recombinant band of 436 bp (Table 9; 742Fwd + P2). In total, our screening procedure conducted on 21 random segregants lead to the isolation of six different homozygous plants for the insertion LORE1 event into the third exon of the 608.1210/LjNPF4.1 gene. These plants were first propagated *in vitro* to ensure maintenance of the screened plants and then transferred to *in vivo* conditions. Plants were first transferred on a closed vermiculite:sand environment and once they fully matured, to soil. Leaf tissues were taken as samples (~10 mg), for total RNA extraction that was used for cDNA reactions. As one of the primer used for the screening

procedure on genomic DNAs was designed from an intron sequence (Fig. 28) we used for this expression analysis a new couple of primers designed on exons 1 and 3, flanking the insertion element (Table 9; P1Frw + P3Rev). As expected the insertion events in the third exon of the LjNPF4.1 gene leads a knock out phenotype in all the homozygous plants. The representative RT-PCR analysis shown in Fig. 28C indicates a knock out phenotype in four homozygous plants (A2, F, D, F2, N), while as expected the heterozygous plant C as well as the wild-type plant amplify the wild type band of 592 bp.

Four out of the six homozygous screened plants produced flowers *in vivo*, successfully producing a new generation of seeds allowing us to proceed with phenotypical characterization. It is important to note that these plants are homozygous for the insertion event in the LjNPF4.1 locus, while the other nine LORE1 inserts of the 30000742 line segregate in an independent way through the generations. This is why we conducted the following phenotypical analysis on the progeny of different LjNPF4.1 knock out segregants as these are not isogenics for the other LORE1 elements distribution.

Once seeds of plants knock-out for LjNPF4.1 gene were obtained through selection and propagation of homozygous LORE1 plants, it was possible to study any phenotypical change incurred by loss of this putative nitrate transporter. Experiments were performed, with knock-out and wild-type seedlings at the same stage of development (Fig. 29). LjNPF4.1 knock-out seedlings seemed to have a slower germination than their wild-type counterparts (data not shown). These seedlings development was followed over two weeks in different nitrate conditions (2mM, 5mM and 10mM KNO_3), their root growth kinetics, secondary and primary root development followed up during this time. After two weeks, plants were sacrificed and shoot weight (dry and wet) and length measured, as well as dry root weight (Fig. 29 and 30).

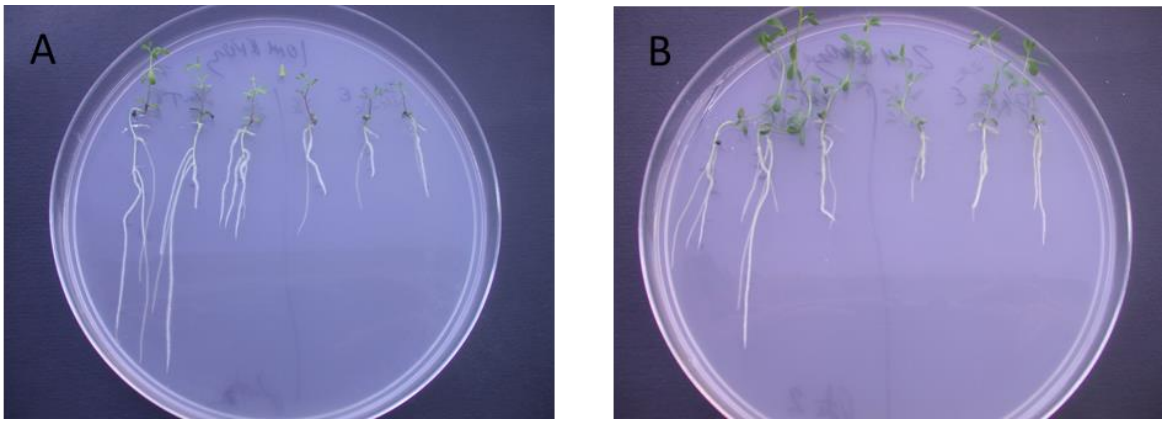


Fig. 29: Plant development of wild type and *npf4.1 Lotus japonicus* plants at different nitrate conditions. A) WT (left) and knock-out (right) plants growth over 2 weeks in 10 mM KNO_3; B) WT (left) and knock-out (right) plants growth over 2 weeks in 2 mM KNO_3.

Loss of the LjNPF4.1 gene determines both a root and shoot development-impairing phenotype that seems to be true even under different nitrate conditions as shown in Fig. 29. In particular, the performed quantitative measuring indicated the both primary root length and number of secondary roots were strongly reduced in the knock out homozygous plants at 2 and 10 mM (Fig. 30; about 45/50 % and 32/38 %, respectively). This defect of the root system was also confirmed by the data of the dry root weight analysis, showing a 43/53% reduction in the knock out LjNPF4.1 when compared to *wild-type* plants (Fig. 31 D-H). The same defect was quantitatively confirmed by the shoot values where knock out plants showed clear-cut deficiencies when compared to *wild-type* plants. The shoot length of the mutant was 67% and 77% of wild type plants in 2 and 10 mM conditions, respectively (Fig. 31 A, E). The shoot fresh and dry weight values were about 55% lower in the mutant in both growth conditions (Fig. 31 B, C, F, G). These phenotypes were observed in independent LjNPF4.1 knock out segregants (data not shown) and, consistently with the uptake activity induced by LjNPF4.1 in Xenopus oocytes, match an inadequate nitrate transport activity function, resulting in inadequate defective growth and development. It is relevant to point out that these root and shoot phenotype studies were also performed in plants at the nitrate concentration of 100 µM (data not shown), but no difference at all was found between *wild-type* and LjNPF4.1 knock-out plants in those conditions in terms of root or shoot development, thus suggesting a main physiological role in planta in the uptake of high

external nitrate concentrations, consistently with the data obtained in the Xenopus oocytes experiments.

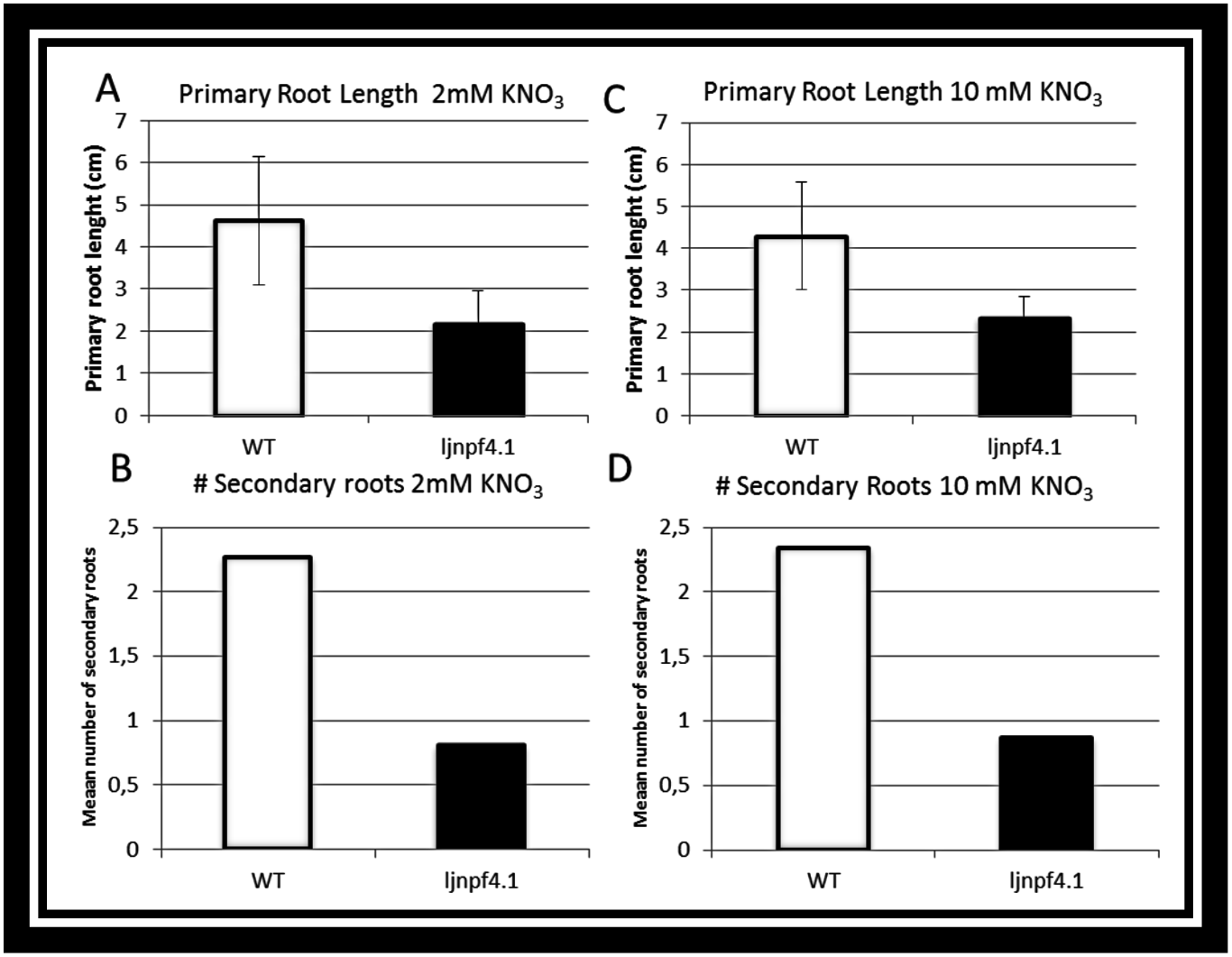


Fig. 30: Root phenotypes of wild-type and *npf4.1 L. japonicus* plants. Primary root length (A); and the number of secondary roots (B) after growth in 2mM KNO_3 for 2 weeks. Primary root length (C); and the number of secondary roots (D) after growth in 10mM KNO_3 for 2 weeks . Data bars represent means and SD of measures performed from two different experiments (10 plants per condition)

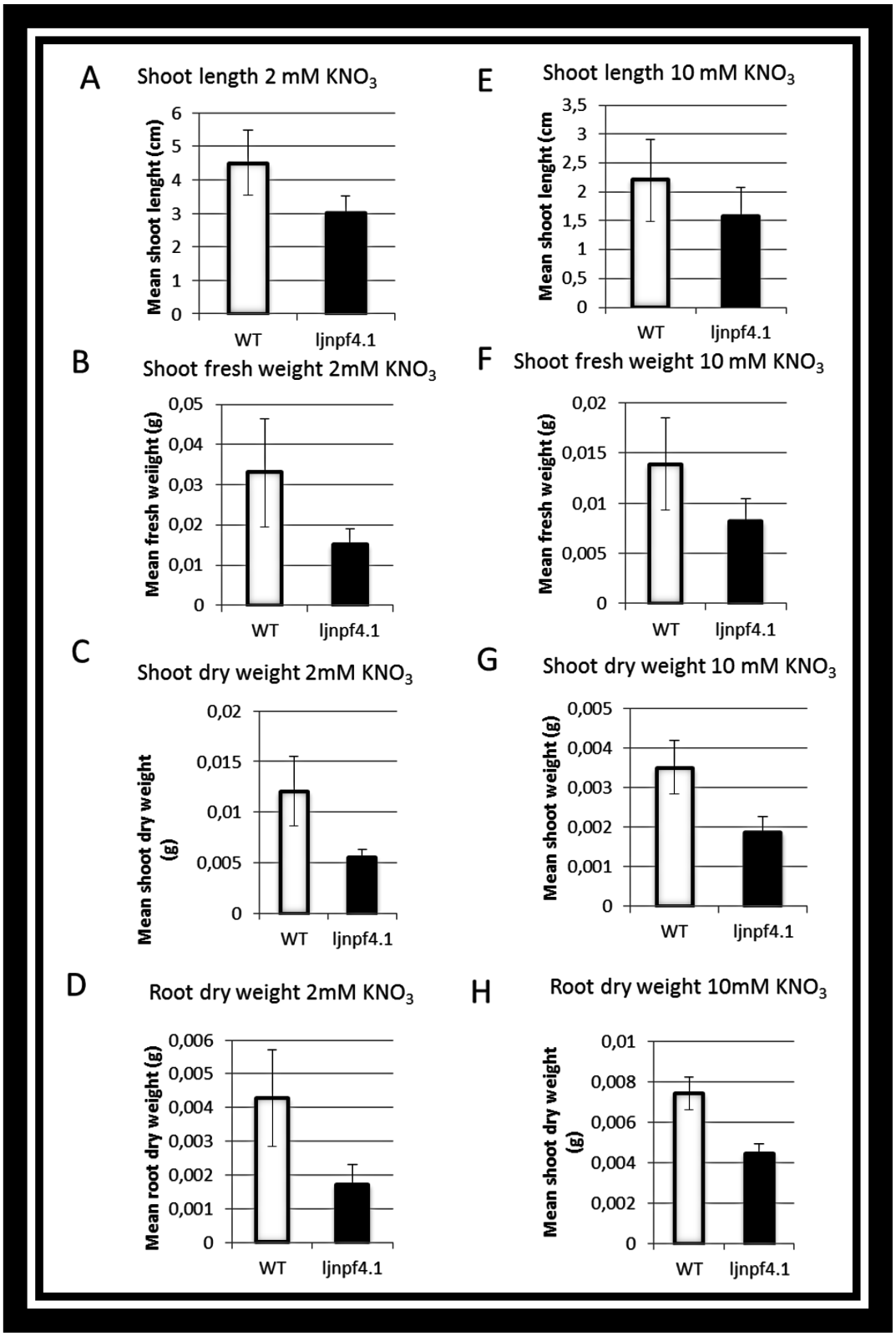


Fig. 31: Shoot phenotype of wild-type and *npf4.1 L. japonicus* plants. Shoot weight (A), shoot fresh (B) and dry (C) weight and root dry weight (D) in plants grown for 2 weeks in media containing 2mM KNO_3. Shoot weight (E), shoot fresh (F) and dry (G) weight and root dry weight (H) in plants grown for

2 weeks in media containing 10 mM KNO_3. Data bars represent means and SD of measures performed from two different experiments (10 plants per condition).

4.4 Low nitrate-induced transcription factors: AP2/EREBP family and LjRAP2.4

Ethylene's role in the regulation of root hair formation and elongation alongside auxin synthesis is well reported in Arabidopsis. In legumes, ethylene is also known for its repressor effect upon nodule organogenesis; as such, any molecular actor that might be related to ethylene-mediated signaling and/or regulation is a worthy candidate to study for their role in nitrogen symbiosis. The AP2/EREBP family is a large family containing various such proteins, reported to possess ethylene-binding motif and playing a role in processes mediated by ethylene in Arabidopsis; perhaps these proteins orthologues in legumes can also play a role not only on the same metabolic processes as in Arabidopsis, but also regulate nodule formation.

In the aforementioned work by Omrane et al. (2009), an affymetrix analysis in *L. japonicus* led to the identification of a pool of sequences whose expression was significantly up- or down-regulated by low (10 µM NH_4NO_3) vs high N (10 mM NH_4NO_3) condition. The chip analysis allowed to report general metabolic differences between Lotus plants grown in low-N and high-N conditions, with repression of genes involved in the nitrogen assimilation pathway suppressed in low-N, as well as genes involved in starch and sucrose synthesis and degradation; low-N plants also display increased expression of genes involved in amino acids ex novo synthesis and repression of those related to amino acid catabolism. Low-N conditions also showed an upregulation of phenylpropanoids and phenolics synthesis, as well as flavonoid metabolism, while other biosynthesis pathways are downregulated, implicating a shift from a primary to a secondary metabolism in nitrogen starvation. This establishes the metabolic changes between different nitrogen environments and its regulation. (Omrane et al. 2009). Table 10 is a list of *L. japonicus* genes potentially involved in signaling pathways related to the nodulation process with a N-dependent regulated profile of expression is reported. In particular, that chip analysis identified the orthologue of the *Arabidopsis thaliana* AtRAP2.4 gene, a member of the AP2/ERBP transcription factor family, LjT01F24.60.nd as strongly up-regulated in plants pre-incubated for 10 days on low N vs plants pre-incubated on high N conditions. Interestingly, this LjT01F24.60.nd (hereafter

called LjRAP2.4) up-regulation profile was also observed in plants pre-incubated on high N condition when these are shifted on low N permissive conditions but only after 9 days that was the range of time needed by Lotus plants to re-acquire the full competence for nodulation. In the work of Asamizu et al. 2008, the analysis of transcription factors induced in the early stages of inoculation and nodule organogenesis included RAP2.4 as one of the AP2/EREBP family members induced in the first 3 hours of inoculation, therefore leading to the hypothesis that LjRAP2.4 might be a candidate actor of nitrate-dependent regulation of symbiosis.

Table 10: List of *L. japonicus* of sequences up- or down-regulated by low (10 µM NH_4NO_3) vs high N (10 mM NH_4NO_3) conditions (Omrane and Chiurazzi 2009).

		CombiMatrix data		Quantitative real-time PCR data	
Lotus Affimetrix ID	Putative annotation	Low N0 vs High-N0	High-N9 vs High-N0	Low N0 vs High-N0	High-N9 vs High-N0
Transcription factors					
chr2.CM0168.430.nd	TAZ zinc finger protein	0.197			
LjSGA_032996.1	**bZIP family protein**	**0.206**	**0.423**	**0.38 ± 0.025**	**0.387 ± 0.025**
LjSGA_085595.1	Scarecrow-like protein	0.234	0.43 + 0.03		
chr2.CM0021.170.nc	HB-Leucine zipper protein	0.306			
Chr1.CM0017.80.nc	NAC family protein	0.342			
Chr5.CM0299.460.nd	Jumonji protein	4.148			
Chr3.CM1144.80.nc	Zinc finger protein	3.408			
Chr1.LjT23D08.90.nc	Lob protein 41	3.046			
LjSGA_020116.2	Scarecrow-like protein	2.961	1.9 + 0.12		
Chr2.CM0608.10.nd	**AP2/EREBP, LjRAP2.4**	**2.795**	**2.063**	**3.016 ± 0.21**	**2.754 ± 0.27**
Chr4.CM0680.260.nc	NAC family protein	2.736	3.03 + 0.23		
LjT01F24.60.nd	ERF/AP2 protein	2.613			
Signalling factors					
Chr5.CM1439.60.nd	Kinase protein	0.09			
LjSGA_025135.01	Kinase protein	0.173			
LjT45M09.90.nd	Kinase protein (CIPK5)	0.186			
LjT06J23.10.nd	Kinase protein	0.208			
Chr4.CM0501.270.nd	Serine/threonine phosphatase	0.331	0.5 ± 0.7		
Chr1.LjT08C17.20.nc	Ca^{2+} dep. Kinase (CDPK1)	0.344			
Chr3.CM0282.970.nc	Phosphatase (LjNPP2C1)	0.371			
Chr1.CM0104.190.nc	**Apyrase, Nod factor binding**	**4.246**	**2.321**	**7.4 ± 0.55**	**3.952 ± 0.42**
Chr5.LjT18P14.20.nd	**Carbox. kinase, *Lj*PEPC-PK**	**3.841**	**4.106**	**4.103 ± 0.1**	**4.742 ± 0.21**
Chr1.CM0816.370.nc	Ca^{2+} dep. Kinase	2.559			
LjSGA_021989.2	Phosphatase protein 2C	2.661			
LjT02O17.60.nc	Ca^{2+} dep.Kinase (LjCCAMK)	2.73			
LjSGA_007864.2	Phosphatase protein 2C	3.017			
LjSGA_129099.1	Cointegrator signal ASC-1	3.106			
Chr3.CM0111.260.nc	GF14 mu protein (GRF9)	3.504			
Biotic and abiotic stress					
CM1983.110.nc	**Threalose phosphatase**	**0.123**	**0.474**	**0.256 ± 0.04**	**0.485 ± 0.06**
LjSGA_106569.0.1	Chaperone-DNAJ heat shock	0.131			
LjT39M07.20.nd	LEA family protein	0.25			
LjSGA_037677.1	Universal stress protein	0.27			
LjSGA_017485.1	**Xylose Isomerase**	**0.274**	**0.484**	**0.345 ± 0.08**	**0.462 ± 0.06**
Chr3.CM0091.150.nc	Cold acclimation protein	0.295			
Chr5.CM0260.550.nd	Chaperone protein	0.299			
LjSGA_016350.2	Nucleotide diphosphate	0.319			
Chr5.LjT15D05.90.nd	Transferase family protein	2.826			
Chr4.LjT06B21.160.nd	UVB-resistance protein	3.101			

4.4 *LjRAP2.4* profile of expression

Asamizu et al. 2008 expression analysis pin-pointed that LjRAP2.4 transcript was induced very early after M. loti inoculation (3 hours) and then its expression decreased during nodule organogenesis. We decided to test the amount of LjRAP2.4 transcript over the various stages of nodule organogenesis in order to better characterize LjRAP2.4 expression. Samples were collected from infected roots at various time points: immediately before inoculation (T0), 24 hours later, 72 hours later and both young 2-week nodules and mature 3-week nodules. RNA was extracted from these samples and cDNA was synthetized for all of these time points. Using this cDNAs, it was possible to study the relative expression of LjRAP2.4 over the course of the nodulation process through PCR. Below are indicated the oligonucleotides and PCR protocol used for this expression analysis (Fig. 32 and Table 12 and 11).

ATGGCAGCTTCAATGGATTTCTACAACAGTTCAACAACTCAACTTCAATCAGATCCCTTTAGT
GGTGGTGAGTTGATGGAAGTTCTAGAGCCTTTCATGAAGACTAGTCCTTCCTCAACTCCCTCT
CAATCTTCTTCATCCCCTTTCTCAAATTCATTCCTACCTTCTTCCAACTCTACCTCTACCTCTTCT
TACCCTTCTTCTCCTTCTCCTTCTCCTTCTCCTTATCCCTTCTACCAAAACCAACCCTCTTCTTCTT
TCTCTTCTTCTTCTTCTTCTTCTTCTTTCTTCACTACACAGCCCAATTTCTACACAGATGGTT
GCTCTTCCATGATGCCATACCTATTTCCCTCAGGGTTTTCATCATCATCACAAAGCCAAAACAA
CTCCATAGGCTTTGAGCAAGAGCAACCAAGTTCTGTTATTGGGCTAAACCAGTTAACCCCATC
TCAGATTAGCCAAATCCAAACCCAGATCCATTTCCAGTCCCAGCAGAACACCAGCAACTCTCT
TAGCTTCCTCGCGCCGAAGCCGGTCGCAATGAAGCAATCTGGCACCCCTCCTAAGCCTACCA
AGCTCTACAGAGGTGTGAGACAGAGGCATTGGGGGAAATGGGCTGAGATTAGGCTTCCCA
AGAATCGGACCAGGCTCTGGCTTGGGACATTTGAGACTGCTGAAGAAGCTGCTCTGGCTTAT
GACAGAGCAGCTTACAGGCTCAGAGGTGATTTTGCAAGGCTGAATTTTCCAAACTTGAAGG
ATCAAGGTTCTTGTTTTGGAGATTACAAGCCTCTGCATGCTTCTGTTGATGCTAAGCTTGATG
CTATTTGTGAGAATTTGGCTGATTTGCAGAAACAGGGGACTAAAGCAGAGAAGGGTGTGAA
GTCTTCTAAGAAAGGTTTGAAGAAACAGGTTCAGGCAGAGGTTGAGAACAAGGTGGAAGCT
TCTTTGTCTCCAGTGGTGACTGAGAGTGAAAACTCTGATGATTCTTCTCCTTTGTCTGATCTTA
CATTTGGTGATTTCAGTGAGCCTCAGTGGGATGCTACTACTTCAGAATATCTTAATCTGCAGA
AGTTTCCATCTTATGAGATTGATTGGGATTCTCTG**TGA**AGTTGAAGTTGAAGTTGAATTTGGT
AGAGTCTTGTTATGTGACTAGTGTTAATGTAGGTGGTAGCTAGTGTCTCTTCTTATCTTGTGG
CTGCTGCAATTGAGTTTTTGAAAATTGCAGTGCTGCATGTAGGATTTATTTGGGTAATTGGGT
TTATGGTTTAGGGTTATTTTTCTTTTTATTATTGATTATTATTATTATGTCAATATATGATGTAA
ATCTCTGTCTACCTTGGTCAGCTGGTTTTTTTCTTGCTTGACCATGGAGAATGACTCCGGTTTA
GGTGTTCTTTGAAGTATATTATTATTGTTATTGATGTAATTCAATTCCAGTTGTATTTCAACTT
AAG**AATATAAT**GTACTACTACTAGTTTGTCTATGGTTATAGATTAATGGTGGTTGTCTCTTCT
TGTTCTTATACTAGCTATCAACTTTTGCTTAACTTAGTTTCTTAGTGTGTTTTTGGAAATTCAG
AATGTGCATACAATTCAATTTTTACAATGAAAGTTTAAGTACTTTGATAGGACCGAATGGAG
GATG

Fig. 32: Coding and 3' UTR region of *LjRAP2.4*. In red is indicated the amplified RNA interference target region. In blue are indicated the primers used for amplifying the region for the overexpressing T-DNA construct. The primers used for the expression analysis are underlined. In green is indicated the predicted polyA region of LjRAP2.4. ATG and TGA are indicated in bold.

Table 12: Different oligonucleotides used for LjRAP2.4 expression analysis.

Name	Sequences (5' to 3')	T_m (ºC)	Amplicon size (bp)
LjRAP2.4Frw	CCTCTGCATGCTTCTGTTGA	55	132
LjRAP2.4Rev	TGCCTGAACCTGTTTCTTCA	54	
LjUbiFrw	TTCACCTTGTGCTCCGTCTTC	64	90
LjUbiRev	AACAACAGCACACACAGACAATCC	70	

Table 13: PCR conditions for RAP2.4 expression analysis.

Step	Temperature (ºC)	Time	
	LjRAP2.4		
Denaturation	94	5 min	
Denaturation	94	30 s	x30 cycles
Annealing	53	30 s	
Polymeralization	72	30 s	
Polymeralization	72	10 min	

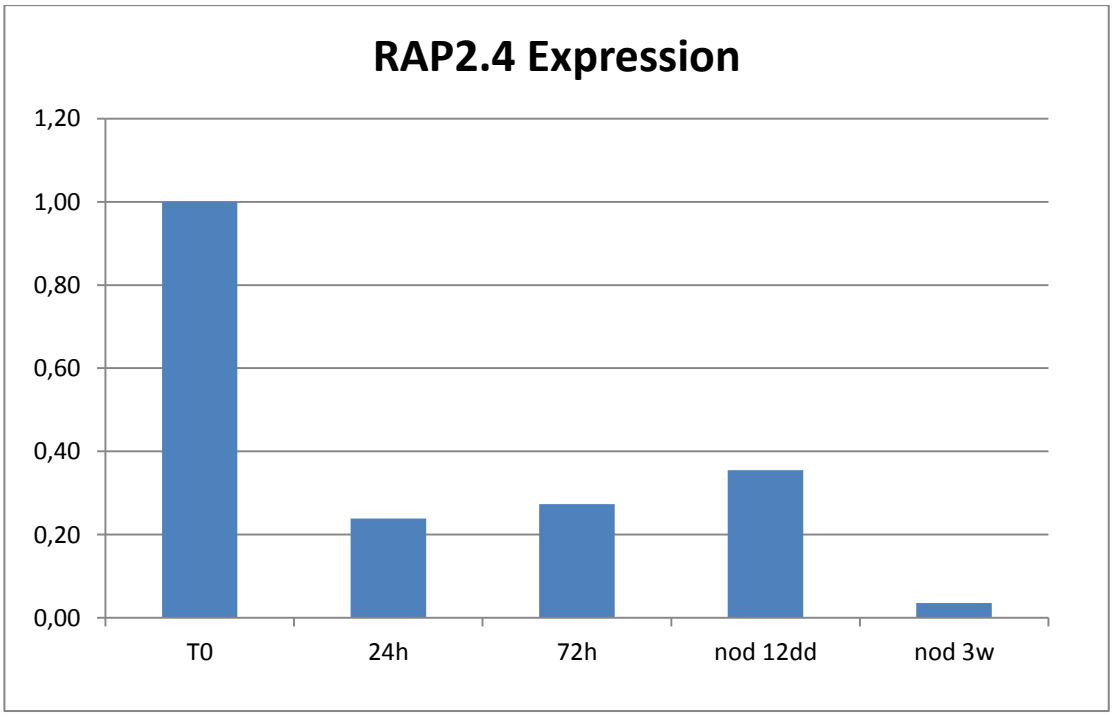


Fig. 33: *Lotus japonicus* RAP2.4 expression in roots and nodules after inoculation with *M. loti.*

The results shown in Fig. 33 seem to match the expectations due to the findings of Asamizu et al. (2008), with LjRAP2.4 primarily expressed in roots, with its transcripts decreasing progressively starting from 24 hours after inoculation (Fig. 33). In particular, while there is still a significant expression in young nodules, RAP2.4 barely seems to be expressed in mature nodules. Therefore, the early 1.9 fold induction at 3 hours post inoculation reported by Asamizu et al. (2008) and our complementary analysis, suggests a role of LjRAP2.4 confined to the very early steps of the nodulation program with LjRAP2.4 being not essential for proper nitrogen-fixation nodular activity.

4.5 LjRAP2.4 functional characterization

4.5.1 Preparation of overexpressing and RNAi T-DNA constructs

In order to investigate the role played by LjRAP2.4 in the nodulation signaling, we decided to exploit transgenic plants with a de-regulated profile of LjRAP2.4 expression. Unfortunately, in this case there were no available LORE1 tagged lines in the gene of interest, forcing us to develop an alternative strategy based on the construction of appropriate T-DNA constructs and generation of *L. japonicus* transformants. The two exploited plasmids were: an overexpressing one (PCAMBIA-LjRAP2.4) and an RNAi one (pB7GWIWG2(II)-LjRAP2.4). PCAMBIA-LjRAP2.4 was obtained through amplification of the 1485 bp fragment including the 1110 bp of the whole coding region (with the TGA stop codon) and 375 bp of the 3' regulatory region (with the predicted polyA site). The designed primers are indicated in the Fig. 32 and include the recognition sites of the *Bam*HI and *Sac*I restriction enzymes (Table 14). The amplification conditions are reported in the Table 15 and the amplified fragment was double digested with *Bam*HI and *Sac*I and ligated into the *Bgl*II-*Sac*I double digested pCAMBIA 3300 vector (Fig. 34). In this T-DNA vector, the control of the expression of the LjRAP2.4 cassette is associated to the CaMV (Cauliflower Mosaic Virus) regulatory sequences: the promoter P35S and the terminator pA35S. The pCAMBIA is a binary 9.5 kb vector which is usually used to allow the expression of transgenic plant sequences. As such, like in other binary constructs, there are "left border" (LB) and "right border" (RB) regions, which are recognized by the *vir* genes of Agrobacterium, which confine the sequences integrated into the plant genome, containing also the hygromycin resistance

cassette used for plant selection (under control of the constitutive promoter 35S CaMV). The gene responsible for resistance to the antibiotic kanamycin allows the selection of pCAMBIA-LjRAP2.4 in bacteria.

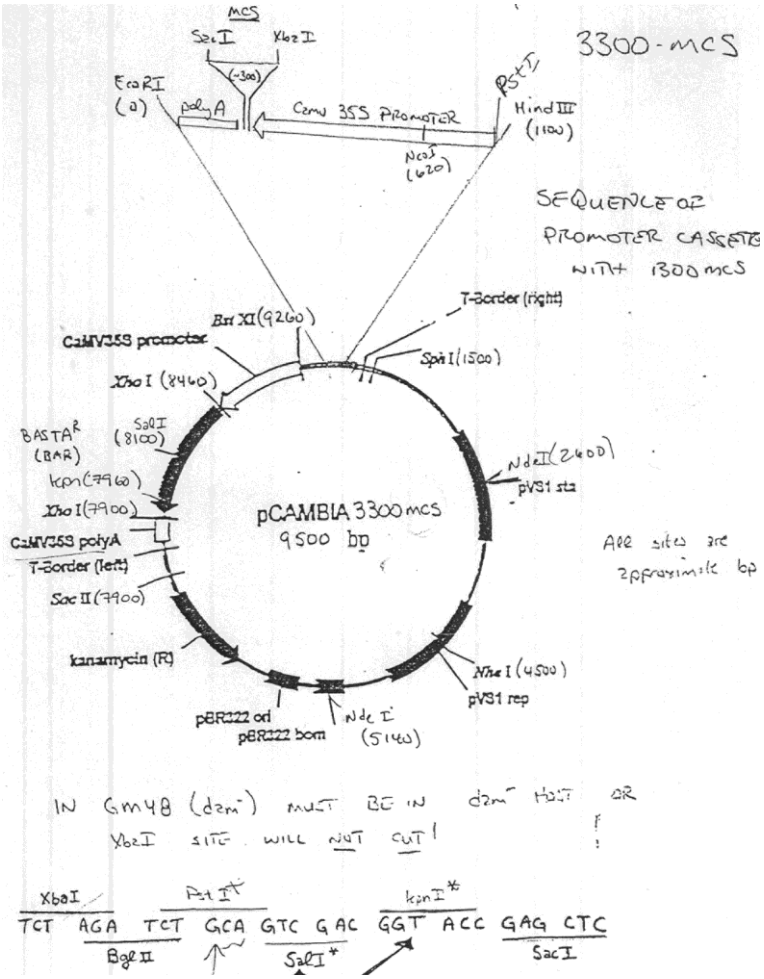


Fig. 34: Map of the pCAMBIA 3300 T-DNA binary vector used for LjRAP2.4 overexpression. The polylinker for the sub-cloning of the expression cassette in indicated in the lower part of the figure. 35S promoter, termination sequences and other features of this vector are also indicated.

The RNAi construct pB7GWIWG2(II)-LjRAP2.4 was obtained through a Gateway recombination cloning strategy (Invitrogen). This recombination technique is based upon the bacteriophage lambda site-specific recombination system, which facilitates the integration of lambda into *the E. coli* chromosome and the switch between the lytic and lysogenic pathways requiring the recognition of attB sequences. A 551 bp sequence including 455 bp of the LjRAP2.4 C-terminal region (including the TGA stop codon) and 96 bp of the 3' UTR region was amplified with specific primers that include the ATTB1 and ATTB2 recognition

sequences (Table 14). The conditions for this PCR amplification reaction are described in the Table 15. An enzymatic reaction catalyzed by the BP clonase was conducted between this PCR amplified fragment and the donor vector pDONR221 (Fig. 35; Invitrogen), carrying the ATTP1 and ATTP2 that recombine with ATTB1 and ATTB2, to obtain an entry vector that can be used for the next reaction. The resulting pDONR221-RAP2.4 construct is an entry vector containing two hybrid sites ATTL1 and ATTL2 flanking the target sequence chosen for driving the LjRAP2.4 RNA interference process.

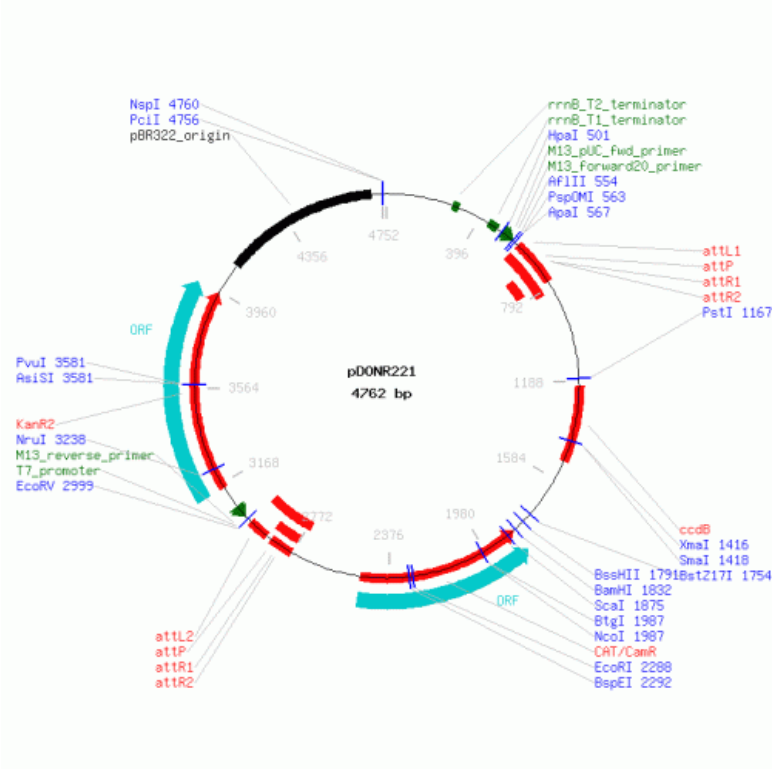


Fig. 35: Map of the pDONR221 donor vector, used to obtain pDONR221-RAP2.4 plasmid after ATTB1xATTP1 – ATTB2xATTP2 site-specific recombination reaction. Restriction sites and crucial features of the plasmids are indicated.

Table 14: Different oligonucleotides used for LjRAP2.4 overexpression and RNAi T-DNA constructs. The target sequences of BamHi, SacI, ATTB1 and ATTB2 are underlined.

Name	Sequences (5' to 3')	T_m (ºC)	Amplicon size (bp)
LjRAP2.4oxFor	GAAGATCTATGGCAGCTTCAATGGATTT	78	1485
LjRAP2.4oxRev	CGAGCTCAGACAACTAGTAGTAGTAC	78	
LjRAP2.4ATTB1For	GGGGACCACTTTGTACAAGAAAGCTGGGTG GGCTTGGGACATTTGAGACT	86	551
LjRAP2.4ATTB2Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTG TGCAGCAGCCACAAGATAAG	86	

Table 15: PCR conditions for amplification of the LjRAP2.4 regions used for overexpression and RNAi T-DNA constructs.

Step	Temperature (ºC)	Time	
LjRAP2.4 overexpression			
Denaturation	94	3 min	
Denaturation	94	30 s	x30 cycles
Annealing	60	30 s	
Polymeralization	72	1 min 30 s	
Polymeralization	72	5 min	
LjRAP2.4 RNAi			
Denaturation	94	3 min	
Denaturation	94	30 s	x35 cycles
Annealing	60	30 s	
Polymeralization	72	1 min	
Polymeralization	72	5 min	

In the second recombination reaction catalyzed by the LR-clonase, the ATTL1 and ATTL2 sites of the entry vector interact with both the two couples of ATTR1 and ATTR2 sequences arranged in an inverted repeat orientation in the destination vector pB7GWIWG2(II)-GUS (Fig. 36). This results in a final expression vector with two couples of ATTB1 and ATTB2 sites. The pB7GWIWG2(II)-GUS is a modification of the pB7GWIWG2(II)-GPF vector, in which the GFP reporter gene was replaced by a *gus*A reporter gene. Since this is a hairpin RNAi expression vector, the RAP2.4 cassette target are inserted as inverted

orientated sequences separated by a plant intron. Expression is driven by the 35S promoter. Spectinomycin and chloramphenicol can be used for plasmid selection in bacteria, while hygromycin is used *in planta*.

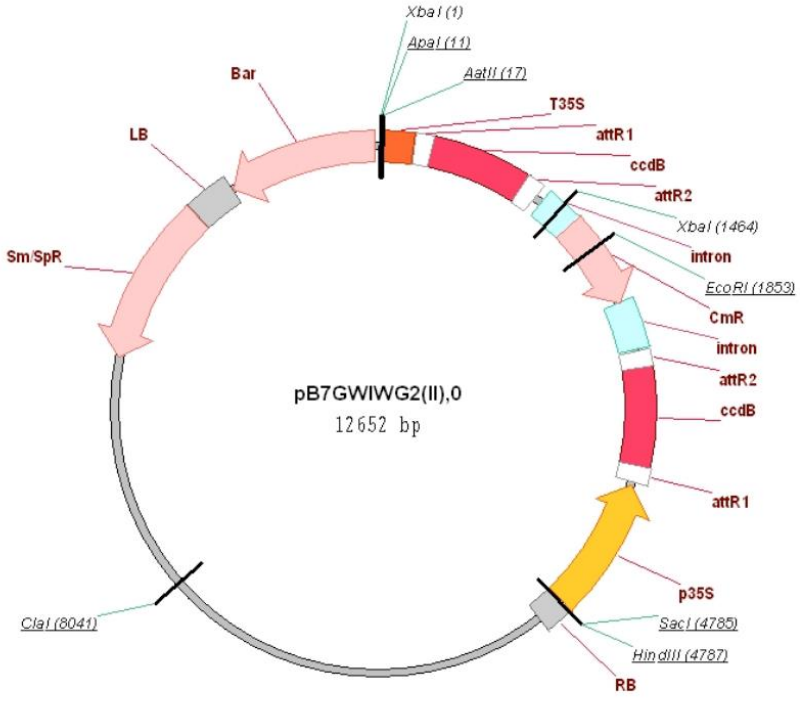


Fig. 36: Map of the pB7GWIWG2(II) destination T-DNA vector. After recombination with the entry vector resulting from the first recombination reaction, it provides us the expression vector. The two couples of ATTR1-ATTR2 sites in an inverted repeat orientation as well as other crucial sequences are indicated.

4.5.2 *Agrobacterium*-mediated transformation with T-DNA

Stable transformed lines for both overexpressing and silencing LjRAP2.4 were obtained, through an *Agrobacterium*-mediated transformation process. *Agrobacterium tumefaciens* AGL1 strain was transformed through electroporation with either one of those two plasmids. Wild-type *Lotus japonicus* plants were grown for 30-45 days in vitro on B5/2 medium, in order to obtain healthy and developed root systems. Those roots were cut and transferred on Petri dishes with CIM (callus induction medium containing 6-BA 0.5mg/l, 2,4-D 0.1 mg/l and sucrose 3%), incubated for 5 days in the same growth conditions as developed *Lotus japonicus* plants. Over these days, because of the action of the large excess of auxins in the CIM, the roots will thicken as consequence of cell proliferation.

After this incubation, roots are cut into small pieces (~0.5 cm) and soaked ten minutes in a liquid culture of *Agrobacterium tumefaciens* carrying our desired construct. The root pieces were squeezed, to maximize the contact surface with the bacteria and for the releasing of the plant aromatic compounds responsible of the *Agrobacterium vir* genes induction. After this step, the infected explants are allowed to co-cultivate with the bacteria for two days, being transferred in new CIM media. After that period of co-cultivation, the explants are rinsed, washed repeatedly and blotted on filter paper, before being transferred into CIM medium supplemented with 200 mg/L cefotaxime; this step arrests the growth of *Agrobacterium*. After 48h and confirmation that Agrobacterium has been eliminated, the selection process starts, with the explants being transferred to CIM medium with 200 mg/L cefotaxime and 15 mg/L hygromycin. Over the course of the next 3 to 4 weeks, all non-transformed material will turn necrotic and has to be removed. In the meantime, photosynthesizing green calli, resistant to hygromycin are selected. The green calli proliferate actively and are supposed to represent the cell progeny of a single transformed plant cell, thus being an isogenic clone.

These transformed calli are transferred on SIM (shoot induction medium supplemented with 200mg/L cefotaxime, 15mg/L hygromycin, 0.5 mg/L TDZ (Thidiazuron phyto-hormone) and 3% sucrose. The reduced auxin/cytokinin ratio of SIM will induce after at least 2 weeks, shoot primordia formation. After that, the explants are transferred into a SIM2 media, which contains a 0.1x TDZ concentration compared to SIM and is hygromycin-free to avoid any further stress condition favoring shoot development. Once the shoot achieves significant dimensions (~1 cm), which allows it to be safely dissected from the rest of the callus, we transfer the shoots into SEM (shoot elongation medium). Once the shoot achieves proper shoots lengths, we cut it and transfer it into RIM (root induction medium). This is a short one week incubation in a medium with a very high auxin (NAA) concentration that consents the root organogenesis after a transient de-differentiation in the basal cut part of the shoot. After this one week incubation, the small transformed regenerant are transferred on REM (root elongation medium), after which they might be transferred from *in vitro* to *in vivo* conditions.

4.5.3 Analysis of seeds progeny and isolation of homozygous plants

Once putative transformants started to produce the next generation of seeds, those seeds were germinated in hygromycin-containing selective medium, in order to select segregating T1 plants carrying our constructs with the hygromycin-resistant gene that segregates in a mendelian way. Then, on the hygomycin selected plants, a molecular analysis to test the effects of the T-DNA constructs on the target LjRAP2.4 gene was performed. Root and shoot samples were recovered, RNA extracted and used to obtain cDNA. Using expression primers for the RAP2.4 gene (Tables 9 and 10), we tested the amount of transcript in the overexpressing and RNAi plants that confirmed the expected increase/decrease of the LjRAP2.4 mRNA in a certain number of T1 plants.

Once we have found plants confirming the de-regulated profile of expression for LjRAP2.4, those were propagated *in vivo*, and allowed to self-fertilize to get a next generation of seeds, in order to obtain T2 plants. This second generation was subjected again to hygromycin selection condition and this procedure was performed on a significant number of seeds (at least 25) to evaluate in a statistically significant way, the segregation of the T-DNA in order to identify homozygous plants where the *hygr* cassette did not segregate anymore. These homozygous T2 plants were identified for two independent transformant lines in the case of the overexpressing plants and only for one transformant in the case of the RNAi plants. These were used for further characterization. In the T2 homozygous overexpressing transgenic lines the LjRAP2.4 gene was analyzed through semi-quantitative RT-PCR, which indicated a 3 and 3.5 fold of induction in the lines oxRapC-4 and oxRapC-6, respectively (Fig 37A); semi-quantitative RT-PCR on the silenced line RNAiRapJ line showed a silencing of LjRAP2.4 expression around 60% (Fig. 38).

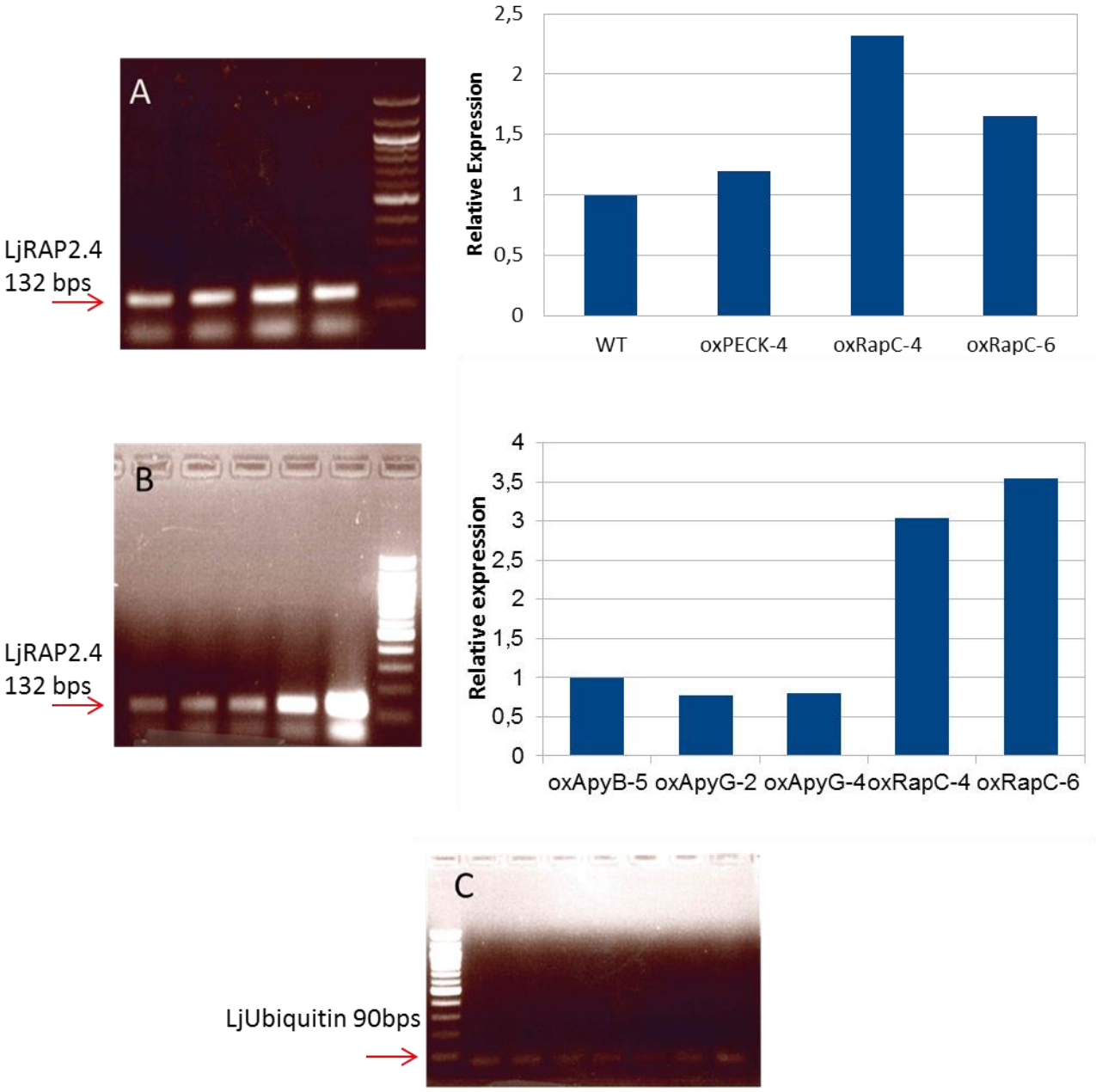


Fig. 37: Relative expression profile of second generation plant roots of the LjRAP2.4 overexpressing lines oxRapC-4 and oxRapC-6. (A) Electrophoresis of the product of the semiq RT-PCR reaction. Expression was analyzed using the LjRAP2.4 expression primers described in Table 10 that amplify a 132 bp fragment. (C) Ubiquitin was used as an internal standard (90 bps). (C) Quantitation of the product of amplification with the first three bars from left representing different hygromycin-resistant lines subjected to the same conditions and used as control (ApyB5, ApyG2, ApyG4).

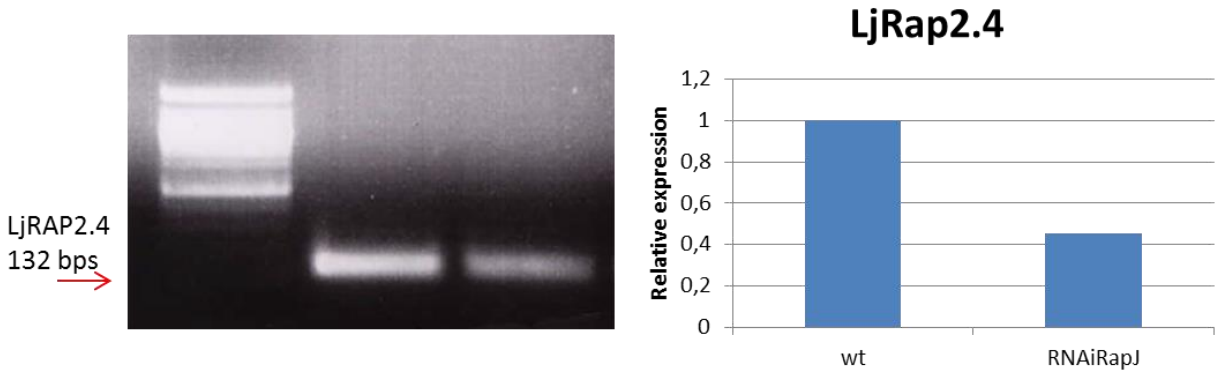


Fig. 38: Relative expression profile of second generation plant roots of the LjRAP2.4 silenced line RNAiRapJ compared with wild-type. Expression was analyzed using the LjRAP2.4 expression primers described in Table 10 that amplify a 132 bp fragment. Ubiquitin was used as an internal standard (90 bps)

4.5.4 Phenotypical characterization

In *A. thaliana* AtRAP2.4 has been associated with various ethylene-dependent phenotypes, including light-sensitive early development phenotypes, being an actor for both hypocotyl gravitropism and cotyledons development. In order to study if LjRAP2.4 was also an intermediary of those ethylene-dependent germination and seedlings early development processes, *wild-type* and RAP2.4 overexpressing plants were germinated to test some of the phenotypes that define the so called ethylene-dependent triple response. The first phenotype to be analyzed was the hook angle of the hypocotyl in plants germinated four days in dark conditions. Lin at al, 2008 reported a slight deformation of the axial "hook" of the hypocotyl in *A. thaliana* plants overexpressing the AtRAP2.4 gene. This phenotype was confirmed in our *L. japonicus* ox-LjRAP2.4 plants where a reduced angle of the hypocotyl was observed when compared to that of *wild-type* plants (Fig. 39) (Lin et al. 2008).

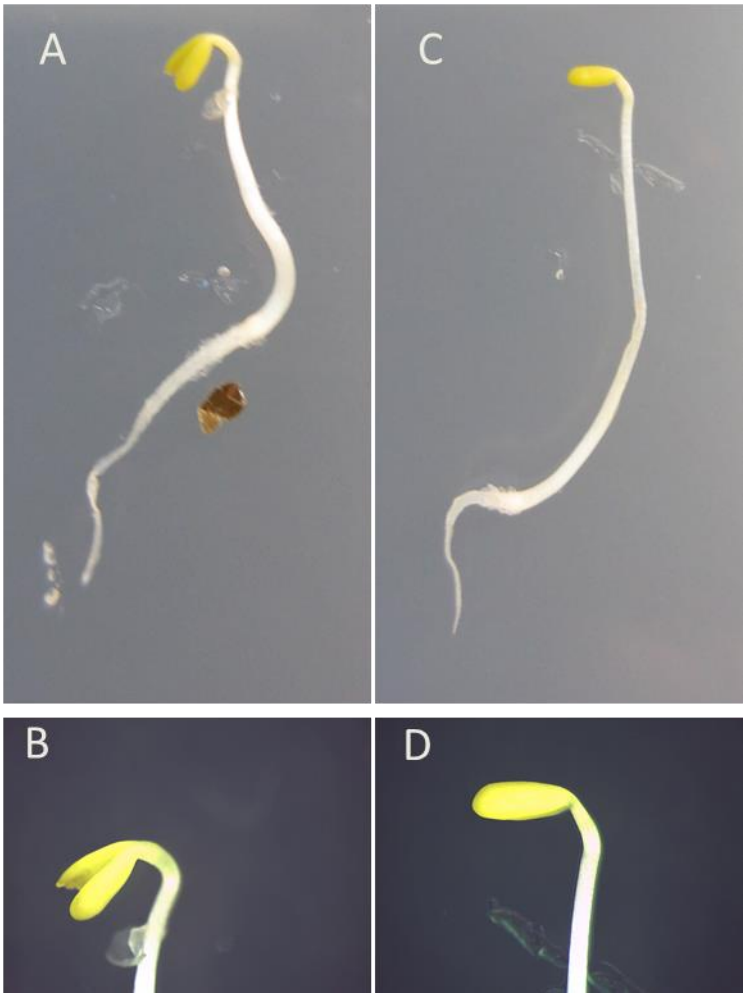


Fig. 39: Representative picture of the angle hook phenotype in *L. japonicus* ox-RAP2-4 plants. A) *Lotus japonicus* wild-type, normal "hook". B) Microscope close-up of the hypocotyl in *Lotus japonicus* wild-type. C) *Lotus japonicus* ox-Rap2.4C ~90º agravitropic "hook" D) Microscope close-up of hypocotyil in *Lotus japonicus* ox-Rap2.4C

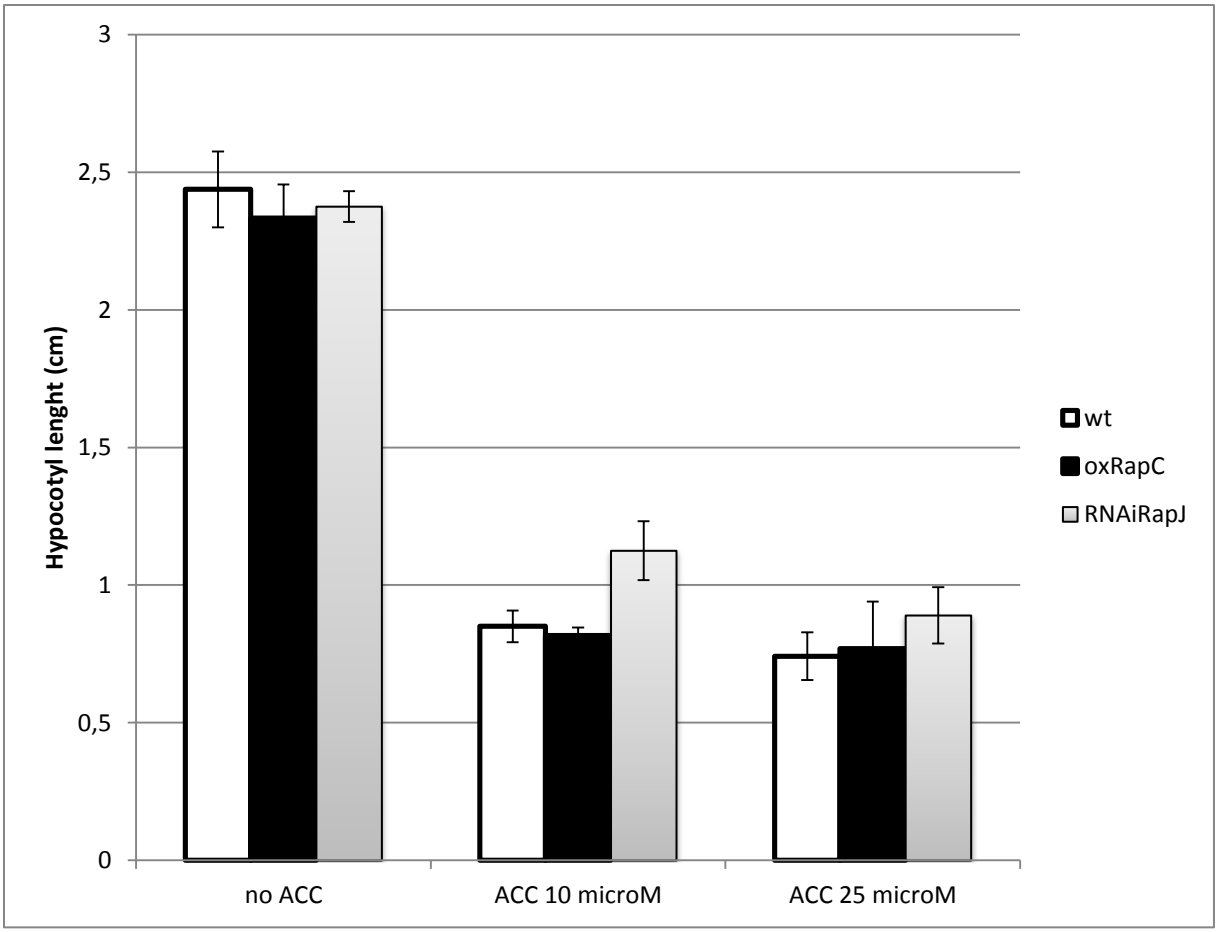


Fig. 40: Hypocotyl length in wt, overexpressing (oxRapC) and RNAi LjRAP2.4 (RNAiRapJ) seedlings maintained in dark conditions for 10 days. The concentrations of ACC used and the meaning of the bars colours are indicated in the legend.

In order to evaluate the role of LjRAP2.4 upon hypocotyl development in darkness conditions and its relationship to an ethylene-dependent phenotype, seedlings of wild-type, overexpressing (oxRapC) and RNAi (oxRNAiRapJ) lines were germinated for ten days in the dark, in media treated with different concentrations (0 , 10µM and 25 µM) of the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC). As expected, ethylene had a negative effect over hypocotyl length at both concentrations (Fig. 40); more remarkable was the increased hypocotyl length the RNAi seedlings compared with the wild-type and LjRAP2.4 overexpressing counterparts at 10µM ACC ($p<0.01$), while such an effect was not detected at 25 25µM ACC. This result was consistent with that obtained with the *Atrap2.4* knock out mutant, suggesting that LjRAP2.4 has an ethylene-dependent negative effect upon hypocotyl length, as reported for the Arabidopsis orthologue (Lin et al. 2008). Therefore, this preliminary analysis indicated a similar role for LjRAP2.4 and AtRAP2.4 in the ethylene-

dependent signaling pathway, which prompted us to study the putative role of LjRAP2.4 in the control of the cross-talk between ethylene and nodulation.

The negative role of ethylene upon the nodule initiation process has been well reported in literature, so we looked into possible symbiotic-related phenotypes. Such an analysis was conducted at the moment only on ox-RAP2.4 plants. Interestingly, when we compared the nodulation response in wild-type and ox-RAP2.4 plants at four weeks after *M. loti* inoculation, we found out that LjRAP2.4 overexpression increased the number of nodules compared to wild-type plants (Fig. 41, E-F). This results position LjRAP2.4 as a positive regulator of early stage organogenesis that matches the reported very early induction of LjRAP2.4 in the first hours upon inoculation with *M.loti* (Asamizu et al. 2008). However, the exact pathway and/or the LjRAP2.4 involvement in the cross–talk between ethylene and nodulation has yet to be proven. A remarkable fact is that the positive effect of RAP2.4 over nodulation seems to be related to nitrate availability in the environment, being more noticeable in plants treated with 1mM and 2.5mM when compared to plants grown on 100 μM nitrate (Fig. 41 D, E and F). This latter observation could represent a link with the up-regulation previously reported in *L. japonicus* plants grown in low N vs high N conditions (Omrane et al. 2009). However the increased number of nodules in 1 and 2.5 mM doesn't impact the shoot phenotypes that is not changed significantly between wt and ox plants (Fig. 41 A, B, C)

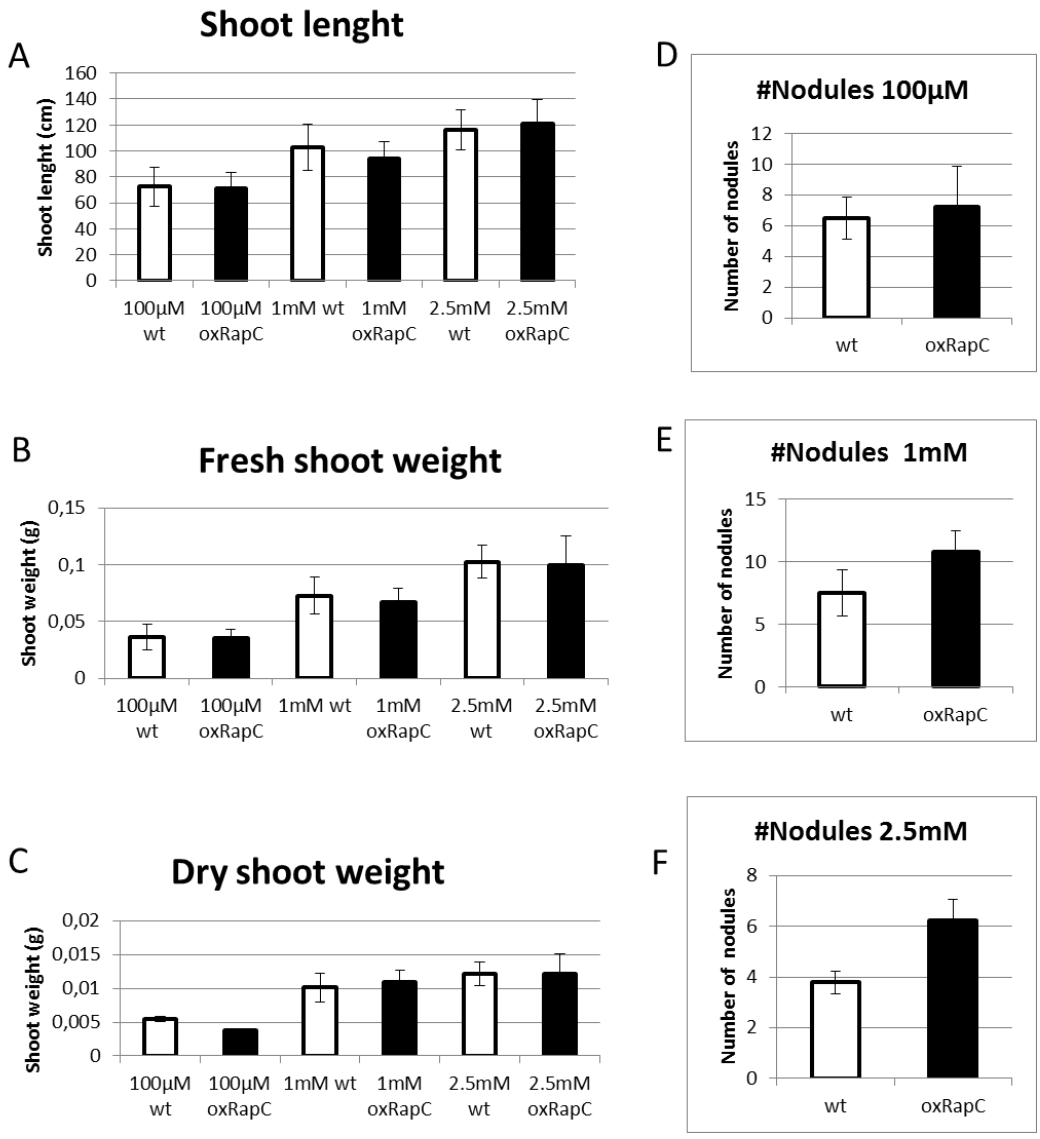


Fig. 41: Root, shoot and nodulation phenotype for *Lotus japonicus* wild-type and overexpressing RAP2.4 plants of the oxRapC line. Fresh shoot length (A), fresh weight B), dry weight (C) of wild-type and oxRapC plants growth at 4 weeks post *M. loti* inoculation (D, E, F) Number of nodules in wild-type and 9C-4 plants at 4 weeks post *M. loti* inoculation. Plants were maintained on 100 µM, 1 mM or 2 mN KNO_3 conditions. Data bars represent means and SD of measures performed from two different experiments (10 plants per condition).

5. Discussion

The plant NPF/NRT1(PTR) family of transporters has been reported to be involved in the transport of a myriad of substrates, playing important roles in the regulation of plant metabolism and in many developmental processes in higher order plants; the homology that the NPF family proteins display with other protein families ubiquitarily distributed across the other major kingdoms of life, fungi, bacteria and animals, showcases pretty well the high importance of the transporters and putative transporters of this family and how evolutionally conserved these motifs and functions are.

In Arabidopsis, NPF family members have been reported to transport a plethora of substrates: AtNPF2.7 (AtNAXT1), AtNPF2.9 (AtNRT1.9), AtNPF2.10 (AtGTR1), AtNPF2.11 (GTR2/AtNRT1.10), AtNPF2.12 (AtNRT1.6), AtNPF2.13 (AtNRT1.7), AtNPF3.1 (AtNitr), AtNPF4.6 (AtNRT2.1/AIT1), AtNPF5.13 (AtNRT1.16), AtNPF5.14 (AtNRT1.15), AtNPF6.2 (AtNRT1.4), AtNPF6.3 (AtNRT1.1), AtNPF6.4 (AtNRT1.3), AtNPF7.2 (AtNRT1.8), AtNPF7.3 (AtNRT1.5) AtNPF1.2 (AtNRT1.11) and AtNRT1.12 have all been identified as nitrate transporters. All of these are low-affinity transporters except for AtNPF6.3. AtNPF6.3 is a complex transporter, having a constitutive expression and transporter function but also being induced in the presence of nitrate in the environment, displaying a pH-dependent nitrate transport activity. AtNPF6.3 multiple roles make it essential to understand the complex nitrate transport system, the different NPF1 low-affinity and NRT2 high-affinity transporters, and how some genes are constitutively expressed while others are induced by environmental changes, showcasing how important the tight interaction between different transporters is required for proper nitrate uptake, adaptation to the environment and preservation of homeostasis (Bisseling et al. 1980; Liu et al. 1999; Guo et al. 2001; Guo et al. 2002; Krouk et al. 2010). Di/tri-peptides transport capacity has been reported for eighteen different *Arabidopsis thaliana* NPF members, such as AtNPF5.2 (AtPTR3), AtNPF8.1 (AtPTR1), AtNPF8.2 (AtPTR5) and AtNPF8.3 (AtPTR2/NTR1), and other family members have demonstrated an affinity for other substrates, such as AtNPF4.1 (AtAIT3), an ABA transporter (Table 2). Arabidopsis NPF have also demonstrated dual transport activity: AtNPF2.9, AtNPF2.10 and AtNPF2.11 can transport glucosinolates in addition to nitrate; AtNPF4.6 transports ABA in addition to nitrate and AtNPF6.3 transports IAA (Table 2) (Okamoto et al. 2003; Chiang et al. 2004; Segonzac et al. 2007; Almagro et al. 2008; Komarova et al. 2008;

Fan et al. 2009; Li et al. 2010; Wang and Tsay 2011; Chen et al. 2012; Kanno et al. 2012; Nour-Eldin et al. 2012; Weichert et al. 2012).

With the Arabidopsis NPF members involved is such important pathways, a myriad of phenotypes have been reported, the whole of them painting a picture of the complexity of nitrate homeostasis. AtNPF2.12 is a nitrate transporter essential for proper germination, with its silencing resulting in embryo deformation and seed abortion, related to defects in nitrogen accumulation within the seeds (Almagro et al. 2008); AtNPF2.13 is a nitrate transporter expressed on the phloem in conditions of nitrogen starvation, being responsible for transport of nitrate for older leaves to developing tissues in response to N deprivation (Fan et al. 2009). AtNPF6.3 has been correlated with chlorate-sensitivity, playing a role in chlorate-uptake, a phenotype that was also confirmed in the AtNPF4.6 transporter (Tsay et al. 1993; Huang et al. 1999). AtNPF6.2 is a constitutive nitrate transporter expressed primarily in the leaf petiole; silencing of this gene results in improper storage and distribution of nitrate in the leaf and results in wider leaves compared to *wild-type* (Chiu et al. 2004). AtNPF7.2 and AtNPF7.3 are two nitrate transporters expressed on xylem, with opposite roles; AtNPF7.3 is responsible for long-distance nitrate transport from root to shoot, while AtNPF7.2 removes nitrate from xylem vessels, balanced against each other to regulate nitrate distribution in response to external stress (Lin et al. 2008; Li et al. 2010). AtNPF1.2 and AtNRT1.12 are two phloem nitrate transporters responsible for nitrate distribution in leaves and silencing of these genes results in disruption of the shoot growth induced by nitrate treatment (1mM and 5mM), marking these molecules as actors in adjustment of nitrate metabolism in Arabidopsis (Hsu and Tsay 2013). AtNPF5.2 is a di/tripeptide transporter NPF member, that is also involved in pathogen defense; silenced mutants for this gene show reduced resistance to *Erwinia carotovora* and *Pseudomonas syringae* and reduced germination in high concentrations of salt (140, 160, and 200 mM NaCl) (Karim et al. 2005; Karim et al. 2007). The AtNPF2.9 mutant *npf2.9* has a most curious phenotype; silenced for a gene responsible for phloem nitrate transport, this mutant has increased plant growth at high concentrations of nitrate (5 and 10 mM), indicating that AtNPF2.9 has a negative effect upon plant growth in high concentrations of nitrate, suggesting that this transporter might control a proper root-shoot distribution of nitrate (Wang and Tsay 2011). Some transporters, like AtNAXT1, have no phenotype associated to

their transport functions, suggesting that they might not play a role in standard culture conditions and/or their roles are redundant and taken over by other transporters (Segonzac et al. 2007).

Criscuolo et al. 2012 study presented various NPF and NRT2 family members of *Lotus Japonicus*, and LjNPF4.1 stood out, with its early induction in roots at low and high nitrate conditions and an increased transcription that was persistent up to 48h after treatment. This nitrate-dependent expression of LjNPF4.1 is specific to roots and was not verified in leaves (Fig. 17). This information lend to the hypothesis that LjNPF4.1 might be a putative dual-affinity transporter, able to uptake nitrate in both high and low concentrations, which was later analyzed through heterologous expression in Xenopus oocytes (Fig. 26). Such an inducible behavior in roots of plants grown in both low and high nitrate conditions is very peculiar among the NPF members and because of the well-known positive and negative effects on legumes nodulation exerted by low and high nitrate external concentration, respectively, this prompted us to further investigate the possible link of the LjNPF4.1 member with the nodule organogenesis control program in response to nitrate. Nodulation is a tight-regulated process that occurs in response to changes of environment; plants are required to adapt to shifts between high and low nitrate conditions and modify their metabolism accordingly. Perception of nitrate conditions, either low or high concentrations, is essential for the trigger of response pathways. which in the case of legumes include the nodule organogenesis machinery and its regulatory pathways. The way nitrate is perceived for the control of nodulation is either as a nutrient or as a signal (Omrane and Chiurazzi 2009). In particular, a nitrate local control on nodule formation that is independent by its assimilation through nitrate reductase reaction has been reported and hence, a molecule with a dual-affinity role could play a key role, acting as a signal sensor and/or transductor ("transceptor") of the external nitrate concentration signal, initiating a signaling cascade and influencing the various nitrogen-symbiosis regulatory actors in a nutritional-independent way. Such a sensing function that is independent by the nitrate transport activity has been already demonstrated for the AtNPF6.3 member that controls the secondary root developmental response to nitrate in *A. thaliana*. Sensing/transducting nitrate conditions is just one of the potential functions of LjNP4.1. Uptake and transport of nitrate from soil to plant, distribution and assimilation has also a *downstream* effect on the nutritional status of

the plant; a dual affinity legume transporter would play an important role in the intricate multi-component nitrate homeostasis systems, involved in nitrate assimilation and thus influencing the plants nodulation auto-regulatory system and conquequently, its demand for nitrogen-symbiosis. Alternatively, a transport activity correlated to auxin, ABA (as already reported for Arabidopsis NPF members) and/or other hormones could represent another possible route of regulation of nodulation involving legumes NPF members. In this thesis the main focus was the generation of a knock out mutant for the LjNPF4.1 member. The phenotypical characterization of the LjNFP4.1 knock out mutant was mainly focused on the analyses of its growth capacity in response to different nitrate conditions. This represents the prerequisite for the deciphering of the biological role in Lotus and to start the analysis of the impact that such a deficiency could have on the efficiency of the symbiotic process.

Looking at the Arabidopsis NPF members with similar characteristics, AtNPF6.3 is a dual-affinity nitrate transporter in Arabidopsis that is expressed in developing regions of roots and shoot, especially during the emergence of lateral roots (Fig. 42), and its expression is, like LjNPF4.1, induced by different nitrate treatments (50 µM and 1mM KNO_3) (Guo et al. 2001; Guo et al. 2002). At *npf6.3* mutant (*chl1*, Fig 43.) presents a deficient root system compared to Arabidopsis wild-type, and while primary root development seems for the most part unaffected, lateral root maturation and elongation is severely impaired. An obvious parallel can be established between AtNPF6.3 role in root development and the root phenotype I report in this thesis for LjNRT4.1: knock-out LjNRT4.1 plants have smaller root systems than their wild-type counterparts, as well as a delay emergence of lateral roots and a decreased number of secondary roots, paired with a stunting of primary root development (Fig. 30). We also report a very significant shoot deficient phenotype in the LjNPF4.1 mutant (Fig. 31). Both the root and shoot phenotypes were particularly prominent in high nitrate conditions (2 mM and 10 mM) as these were not observed at 100 µm conditions. Such a result is consistent with the strong uptake activity observed at 30 mM nitrate concentrations in Xenopus oocytes (Fig. 26). The exact LjNPF4.1 Km for nitrate has not been measured yet and a wider range of concentrations has to be tested before to state that this transporter is capable of nitrate transport in the high affinity range. However the pH dependent transport capacity observed in Xenopus at 1 mM nitrate concentration seems quite significant. Nevertheless, whether the role of LjNPF4.1 would be merely limited to a transport function,

we should not expect a deficient phenotype in the knock out mutant at 100 μm nitrate, as in this range of concentration the main actors in nitrate uptake are the NRT2 high affinity nitrate transporters and hence the potential defect of the LjNPF4.1 would be certainly masked by the uptake activity of the endogenous *L. japonicus* NRT2 proteins. However, AtNPF6.3 does not have shoot phenotype but has a strong expression in developing shoot, which is hypothesized to be due to a direct role of AtNPF6.3 upon shoot development (Guo et al. 2002). Another key difference between AtNPF6.3 and LjNRT4.1 is in their response to auxin: while LjNRT4.1 has shown no transcript changes after treatment with either auxin or cytokinin, AtNPF6.3 is induced after treatment with auxin (Guo et al. 2002; Criscuolo et al. 2012). In the case of AtNPF6.3 this auxin response is directly linked to the additional function of nitrate sensor recently reported (Krouk et al. 2010). In fact, the AtNPF6.3 transport activity is not limited to nitrate, being also able to transport auxin; remarkably, the transport of both auxin and nitrate by AtNPF6.3 is interconnected, as AtNPF6.3-mediated auxin transport occurs only in low concentrations of nitrate (Krouk et al. 2010). AtNPF6.3 role as transporter of both nitrate and auxin lends credibility to the currently accepted model: that in response to a low nitrate environment, AtNPF6.3 regulates root architecture through auxin transport. In the absence of nitrate, constitutive AtNPF6.3, which is expressed in the root tips, facilitates auxin uptake into lateral epidermal cells, lowering auxin accumulation in the lateral tip and repressing lateral root growth; high concentrations of nitrate (~1mM) acts as a signal, which is recognized by AtNPF6.3, inhibiting its transport of auxin and allowing it to accumulate in the lateral root tips, stimulating lateral root growth (Fig. 43). In the *chl1* mutant, nitrate concentration is not perceived and auxin transport is inhibited at both N deprived and KNO_3 treated conditions, hence maintaining the sub-optimal concentration of auxin in the root tip and consequent increase elongation in both N conditions (Fig. 43). Strikingly, the root phenotype of the chl1 mutant is independent by its transport activity as this mutant is not affected in its nitrate transport capacity, hence indication the dual function of transceptor.

As mentioned before, LjNPF4.1 has no auxin or cytokinin-dependent induction. Therefore, although its capability to transport auxin is not known yet, this stringent feature suggests a different role that AtNPF6.3 and the observed growth phenotypes (Fig. 29-31) could be related to a mere nitrate transport deficiency. However, the question of whether or

not this nitrate transport defect might impact the nodulation program efficiency is still an open question and it will be addressed soon.

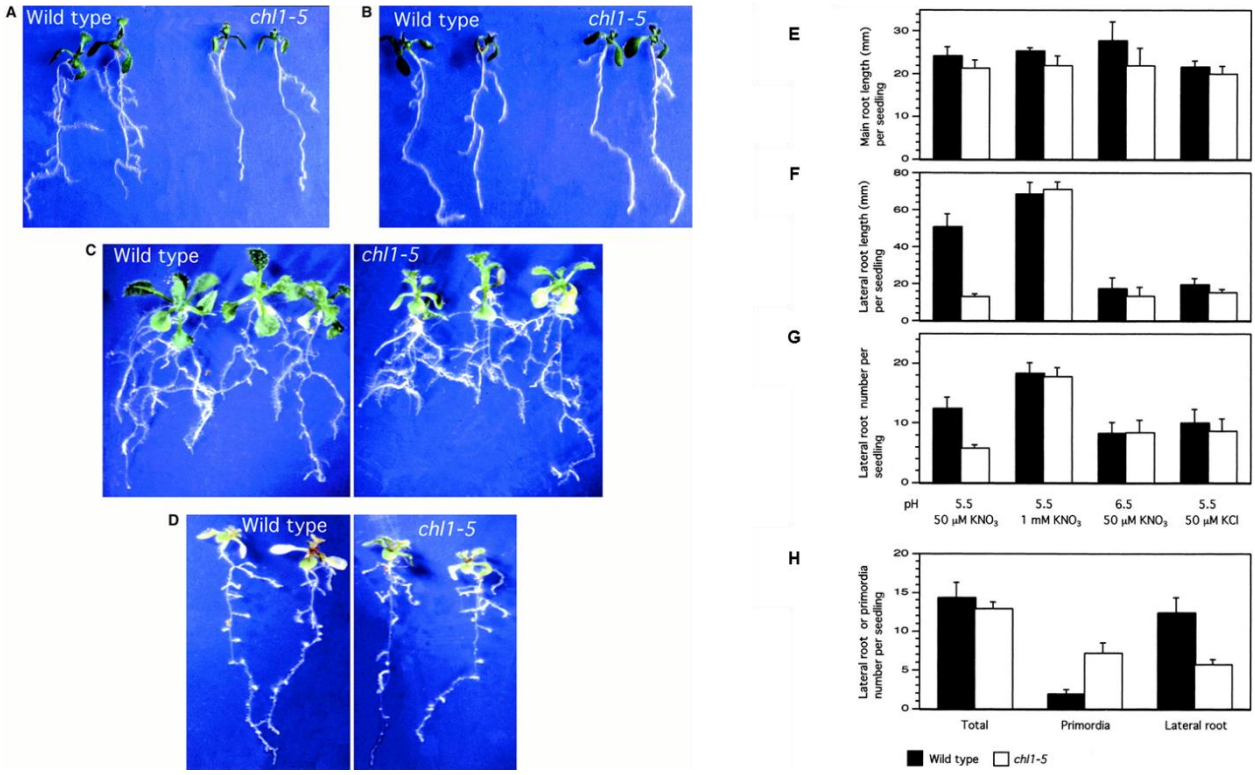


Fig. 42: Main root length, number of lateral roots, lateral root growth in the Arabidopsis wild-type and *chl1* deletion mutants. Five-day-old wild-type and *chl1-5* seedlings 3 days after transfer from germination medium (10 mM NH_4NO_3, pH 5.5) to test media are shown. All seedlings were grown vertically on agarose plates before and after transfer under continuous light at 24°C. Medium in NH_4NO_3 was replaced with the following conditions: 2.5 mM $(NH_4)_2SO_4$ and 50 µM KNO_3, pH 5.5 (A); 2.5 mM $(NH_4)_2SO_4$ and 50 µM KNO_3, pH 6.5 (B), 2.5 mM $(NH_4)_2SO_4$ and 1 mM KNO_3, pH 5.5 (C) and 2.5 mM $(NH_4)_2SO_4$ and 50 µM KCl, pH 5.5 (D). Main root growth (E), sum of lateral root length (F), number of lateral roots (emerged) (G) and number of emerged lateral roots, non-emerged lateral root primordial and the sum of the two (H) (Guo et al. 2001).

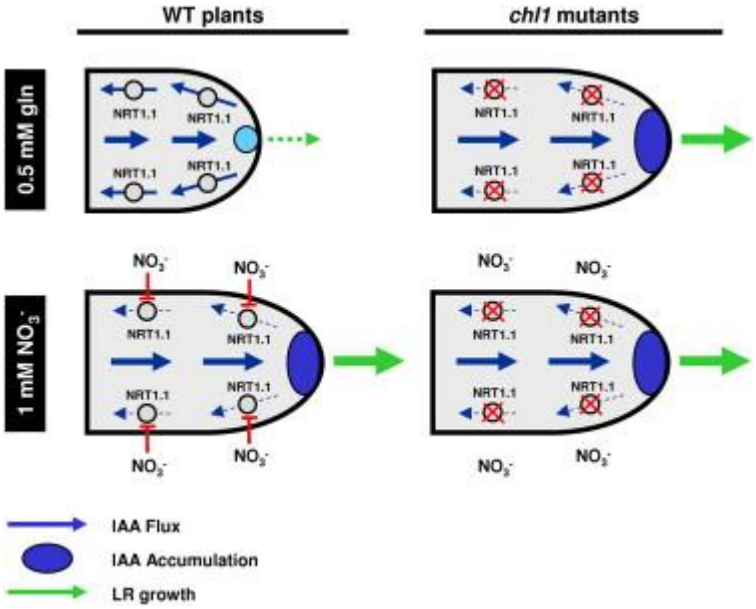


Fig. 43: Schematic Model for AtNPF6.3 control of lateral root growth in response to nitrate. Two situations are shown to illustrate the specific effect of NO_3^- on lateral root growth, corresponding to plants supplied either with 0.5 mM glutamine or with 1 mM NO_3^- (1 mM external N in both cases). The model postulates that in the absence of NO_3^- (glutamine-fed plants), AtNPF6.3 favors basipetal transport of auxin in lateral roots, thus preventing auxin accumulation at the lateral root tip. This slows down outgrowth and elongation of lateral roots. At 1 mM NO_3^-, facilitation of basipetal auxin transport by AtNPF6.3 is inhibited, leading to auxin accumulation in the lateral root tip and accelerated growth of lateral root. Accordingly, AtNPF6.3 mutation in *chl1* plants, which suppresses facilitation of basipetal auxin transport by AtNPF6.3, results in high auxin levels in the lateral root tip and accelerated growth of lateral roots, regardless of the external N source. Direct basipetal auxin transport by NRT1.1 is shown for simplicity to illustrate its facilitation of this transport flow (Krouk et al. 2010).

AtNPF4.6 is another Arabidopsis NPF nitrate transporter that is worth of closer comparison with LjNPF4.1. Unlike both LjNPF4.1 and AtNPF6.3, which have very low level of basal expression and are induced in response to nitrate provision and in the latter case, in developing regions of the plant, AtNPF4.6 is constitutively expressed and does not rely on nitrate-dependent induction (Huang et al. 1999). AtNPF4.6 is primarily expressed in root hairs and root epidermis, a profile of expression similar to our own findings of LjNRT4.1 (Fig. 20), this epidermis and root hair spatial profile being strongly consistent with proteins involved in nitrate uptake (Fig. 44). Transgenic plants expressing antisense AtNPF4.6 exhibited reduced nitrate-induced membrane depolarization and nitrate uptake activities at 10 mM nitrate and exhibited an enhanced resistance to chlorate, a nitrate analogue that is

toxic for plants. AtNPF4.6-injected oocytes kinetic analysis revealed a Km for nitrate of ~5.9 mM at pH 5.5 (Huang et al. 1999). The data obtained up to now revealed a strong uptake in LjNRT4.1-injected oocytes at 30 mM KNO_3 and pH 6.5. While both LjNRT4.1 and AtNPF4.6 seem to have a preferential nitrate uptake activity at high concentrations of nitrate, AtNPF4.6 seems to be able to uptake nitrate in a lower pH than LjNRT4.1. Therefore, although both LjNPF4.1 and AtNPF4.6 seem to be involved in the nitrate uptake into the root form the external soil, the nitrate-dependent induction of LjNRT4.1 suggests for this transporter a role mainly devoted to the necessity of the plant response in a changing environment. Recently, AtNPF4.6 has been reported to be able to transport ABA in addition to nitrate and, its high sensitivity and selectivity for this phytohormone suggests it might be a better transporter for ABA than nitrate (Kanno et al. 2012). Since both in Lotus and white clover, root treatment with ABA reduces the number of nodules after hair root deformation, ABA is likely to be involved in the auto-regulation of nodule numbers, and any ABA transport activity might suggest a strong candidate for nodule organogenesis regulation. As both nitrate and ABA arrest nodulation down-stream of hair root curling, a hypothesis can be suggested about a putative nitrate-induced molecular actor that regulates nodule organogenesis through an ABA-dependent pathway – drawing a parallel to AtNPF6.3 role crossing-over between nitrate/auxin pathways (Suzuki et al. 2004; Barbulova et al. 2007). Despite the spatial profile of LjNPF4.1 and AtNPF4.6 being similar and both being classified in the same clade of the NPF family, there is still no data on LjNPF4.1 transport activity and no statements can be made about its ABA-related role. It is of interest to note that AtNPF4.6 and LjNPF4.1 share the same evolutionary subclade inn the phylogenetic tree reported by Leran et al. (2014), thus suggesting putative common transport capabilities and related functions.

In conclusion, the LjNPF4.1 characterization reported in this thesis, has to be completed through a more detailed phenotypical analysis that will include: The study of LjNRT4.1-mediated uptake of other substracts such as auxin and/or ABA in heterologous expression systems and especially the evaluation of all the possible symbiotic phenotypes (nodule initiation and development). In this way it will be possible to pinpoint the possible role played by LjNRT4.1 in Lotus and whether this is exerted through a mere transport function with the related effects on N metabolism or through a nitrate transducer action.

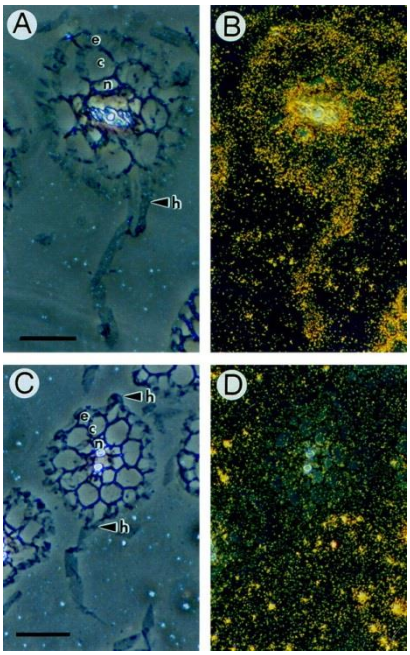


Fig. 44: High level of AtNPF4.6 mRNA reported in root hairs and epidermis. *In situ* hybridization of antisense AtNPF4.6 probes to a cross-section of mature Arabidopsis root tissue, bright-field microscopy (A) and double exposures using a colored filter for the dark-field exposure caused the AtNPF4.6 signals to appear yellow (B). *In situ* hybridization of sense AtNPF4.6 probes to a cross-section of mature Arabidopsis root tissue, bright-field microscopy (C) and double exposures with a color filter for dark-field exposure (D). Legend: c - cortical cells; e - epidermal cells; h - root hair; n - endodermal cells. Bars represent 50 µm (Huang et al. 1999).

There is a rich literature on ethylene and its diverse roles in plant development and metabolism, especially on the model system *Arabidopsis thaliana*. Ethylene is important for the induction of root hair development and auxin synthesis, influencing the auxin tip gradient and thus, regulating root system development through that pathway as cell division increases and there is inhibition of cytokinin-dependent cell elongation/differentiation processes. A high concentration of ethylene commits Arabidopsis atrichoblast to form root hairs, determining cell fate. Beyond root development, ethylene is also responsible for fruit ripening, leaf senescence and flower abscission. (Tanimoto et al. 1995; Cao et al. 1999; Chen et al. 2005; Ortega-Martinez et al. 2007).

In legumes, ethylene functions as a repressor of nodulation, triggering an early arrest of nodule organogenesis by preventing calcium spiking; in addition, ethylene also determined nodule positioning as the silver nitrate treatment of roots that inhibits ethylene synthesis in the protoxylematic poles in front of which nodule emergence takes place,

determines an increased nodule formation in a wrong location in between xylematic and phloematic poles (Heidstra et al. 1997; Ding and Oldroyd 2009). Even with such clear phenotypes, genetic and biochemical characterization of the ethylene-pathway and its molecular intermediaries is still missing. The AP2/ERF transcription factor family derives its name from the ethylene-responsive element binding motifs, and has many members that have been reported as downstream effector elements of various ethylene-dependent pathways and responses in Arabidopsis; AP2/ERF family members are important regulators of floral and leaf development and part of the various mechanism used by plants to respond to various types of biotic and environmental stress (Riechmann and Meyerowitz 1998). In Lotus, some of the members of this family have been reported to be induced in low nitrate conditions, during the early stages of nodulation and after treatment with ethylene and/or jasmonic acid, with LjRAP2.4 being included among said transcription factors, raising the hypothesis that LjRAP2.4 might play an important role in the early stages of nodulation, one that might be ethylene-dependent (Asamizu et al. 2008; Omrane et al. 2009). Unlike Asamizu et al. 2008 we studied the LjRAP2.4 expression (Fig. 33) over important milestones of the nodule organogenesis process, instead of during the early stages of infection. Asamizu et al. 2008 reported that LjRAP2.4 peak expression was on the first three hours after infection, with it decreasing over time; we observed that there was a considerable expression of constitutive LjRAP2.4 in roots, which was significantly decreased in later milestones and into the nodular tissues. These two profiles suggest that LjRAP2.4 might be important in the regulation of the early stages of nodulation but is not required for functional nodules and active nitrogen-fixation symbiosis.

In Arabidopsis, RAP2.4 and RAP2.4B belong to the same sub-class of the AP2/ERF family, being responsible to dehydration, high salinity and cold (RAP2.4) and heat (RAP2.4B); these two proteins also target different aquaporins, suggesting a homeostasis role (Lin et al. 2008; Rae et al. 2011). Arabidopsis RAP2.4 was associated with proper apical hook curvature in darkness; performing similar experiments in Lotus overexpressing LjRAP2.4 plants, we observed the same phenotype reported for Arabidopsis in Lin et al. 2008 (Fig. 39 and 40). This phenotype defines both AtRAP2.4 and LjRAP2.4 light-dependent positive regulators of the hypocotyl hook opening process – one of the various processes of plant development that is influenced by ethylene, establishing LjRAP2.4 as an element of ethylene-induced

pathways just as its Arabidopsis orthologue. The same work of Lin et al. 2008 reported a light and ethylene-dependent influence of RAP2.4 over hypocotyl development; our experiments with overexpressing and silenced RAP2.4 lines treated with 10 µM and 20 µM of the ethylene-precursor ACC confirmed that hypocotyl elongation sensitivity phenotype to ACC in the dark indicated a significantly reduced effect on the hypocothyl length of RNAi silenced plants, placing again LjRAP2.4 as a necessary actor for this ethylene-induced phenotype. The result reported in this thesis demonstrate a negative impact of LjRAP2.4 upon hypocotyl length and this further reinforce the statement that LjRAP2.4 shares ethylene-mediated roles with its Arabidopsis orthologue.

Our analysis of the impact of overexpression of LjRAP2.4 upon nodulation in different nitrate conditions confirmed what the previous findings led us to believe: at 1 mM and 2.5 mM of nitrate, RAP2.4 has a positive regulatory effect upon nodule organogenesis, overexpressing plants developing more nodules than their wild-type counterparts (Fig. 41 E-G). Asamizu et al. (2008) reported that various transcription factors were induced in different stages of nodulation, a list that includes members of the CCAAT, bZIP, C2H2, Homebox, NAC, WRKY, C3H, MADS, C2C2-Dof, CPP and AP2/EREBP families; amongst the AP2/EREBP reported, LjRAP2.4, LjERF1 and LjERF2 were all found to be expressed in the initial steps of nodule formation. A detailed symbiotic phenotypical analysis was conducted only for LjERF1, reporting a positive effect of LjERF1 overexpression when compared to wild type plants whereas *LjERF1* interference strongly inhibited nodules formation (Asamizu et al. 2008). The analysis conducted in this thesis reports a preliminary similar result with the overexpressing LjRAP2.4 plants that develop significantly more nodules than their wild-type counterparts. While in our case we still do not know the exact pathway through which LjRAP2.4 regulates nodule organogenesis, LjERF1 has been hypothesized to increased nodulation events by lowering the early plant defense responses to bacteria, as Arabidopsis ERF1 is known to play a role in ethylene/jasmonic acid-dependent pathogen response and induction of a marker of pathogen response marker (LjPR10-1) occurs in hairy roots silenced for ERF1 (Lorenzo et al. 2003; Asamizu et al. 2008; Omrane et al. 2009). In Arabidopsis, RAP2.4 and ERF1 have been organized in two different subgroups of the AP2/ERF family: AP6 and ERF (Nakano et al. 2006; Mizoi et al. 2012). While both AP6 have members reported to be involved in abiotic stress response and drought-response, ERF family members, which

include AtERF1, have enhanced resistance to fungi and/or bacteria which has not been verified in any AP6 subfamily members (Nakano et al. 2006; Mizoi et al. 2012). As neither AtRAP2.4 nor other AP6 members have been found to be involved in pathogenic defense processes, the same might be true for LjRAP2.4, which would explain the profile differences between LjRAP2.4 and LjERF1: LjRAP2.4 has a more pronounced response to ethylene and jasmonic acid, and *gus*A promotor for LjERF1 activity has been found only in root epidermis and not in any nodule primordial or root apex (Fig. 45), while LjRAP2.4 was expressed in root apex and nodule primordial (Fig. 46). This suggests a different route of action for the control of the nodulation process. The positive effect on nodule numbers in the oxLjRAP2.4 plants when compared to wild type plants is observed in 1 mM and 2.5 mM nitrate conditions, while it is not observed in 100 μM nitrate (Fig. 41). These data have an interesting correlation with what was reported in the work of Omrane et al. (2009) where LjRap2.4 transcription was strongly induced in Low N vs High N conditions. A possible explanation would be that the physiological low N-dependent induction is crucial to reach the LjRap2.4 level required for a normal nodule formation process and therefore only in 1mM and 2.5 mM nitrate conditions where the LjRap2.4 could be reduced, the effect of the overexpression can be observed.

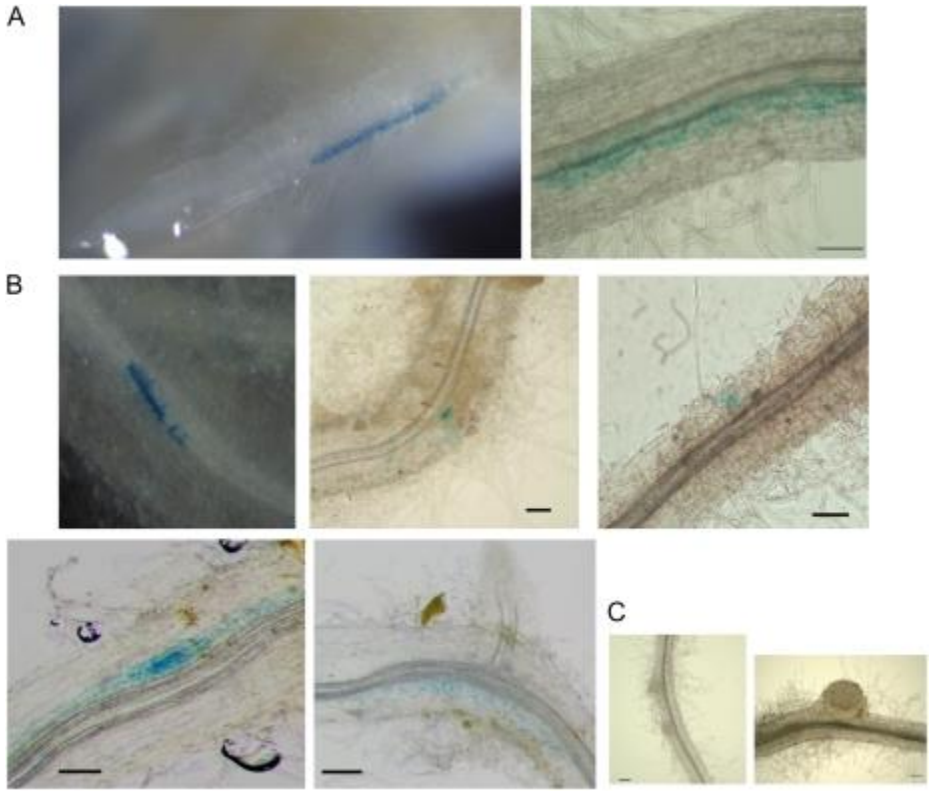


Fig. 45: *LjERF1* promoter:GUS assay after *M. loti* inoculation. A, Histochemical GUS staining was observed in an epidermal region of the*LjERF1* promoter:GUS transgenic hairy root at 3 h after *M. loti* inoculation. B and C, GUS staining at 10 d after inoculation (Asamizu et al. 2008).

LjRAP2.4 promoter activity was studied thought *gus*A-fusion construct (Ricciardi: unpublished data). In this analysis, LjRAP2.4 was found to have strong expression in the meristematic regions, and both nodule and root primordia (Fig. 45). This profile is consistent with results obtained for AP2/ERF transcription factors of *Medicago truncatula*; various Medicago AP2/ERP are expressed primarily on the root apex and associated with plant development and root apex response to stress (Mantiri et al. 2008; Gruber et al. 2009). In particular, the LjRAP2.4 expression in nodule primordia reported by Ricciardi (unpublished data) is consistent with our described phenotype and with the mentioned reports that pinpoint the role of LjRAP2.4 over nodule regulation as occurring early during the nodulation process.

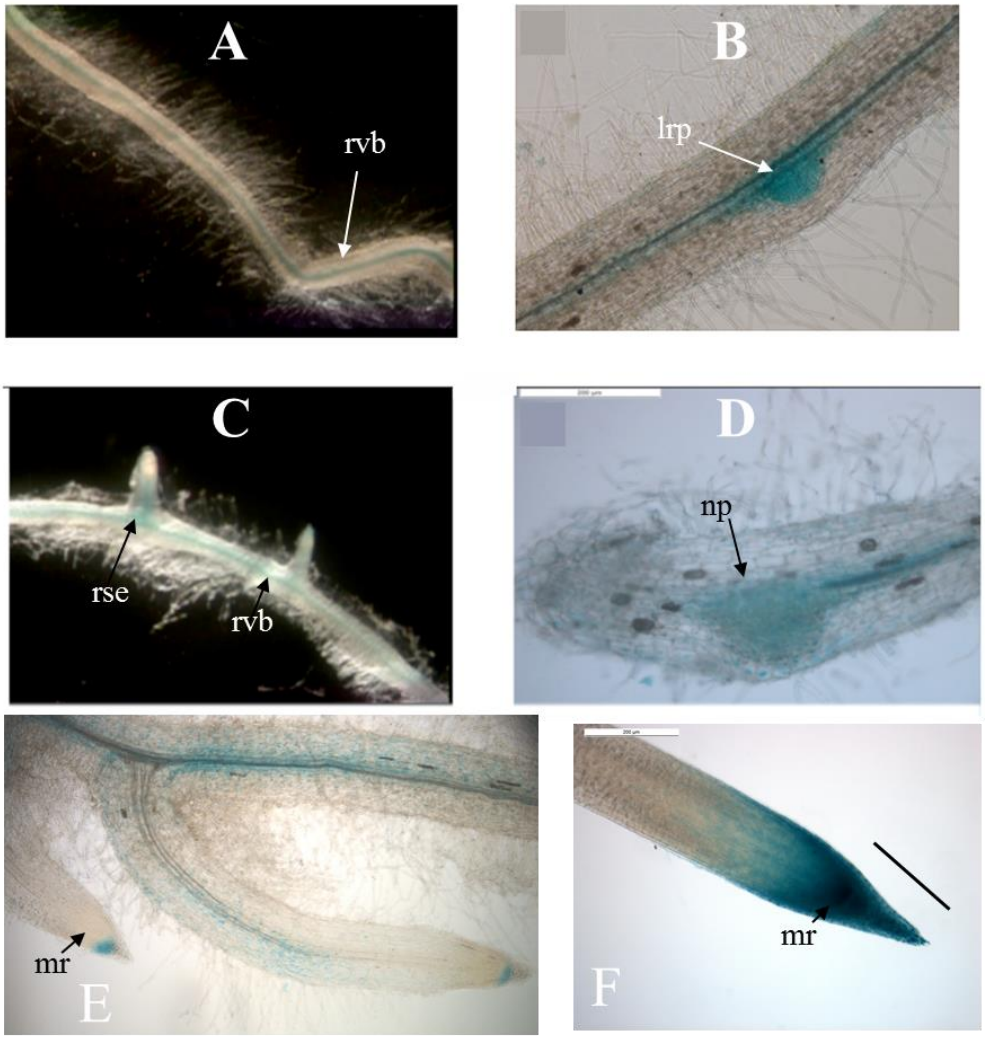


Fig. 46: Analysis of the spatial profile of the fusion construct LjRAP2.4-pr-*gus*A in *Lotus japonicus* hairy roots. Expression in roots (A, C, E), close-up of nodule primordia (B, D) and root apex (F). Legend: rvb= root vascular bundle; lrp= lateral root primordium; rse= root secondary emergence; np= nodule primordium; mr= meristematic region. (Ricciardi: npublished data).

While the current data allows us to speculate that LjRAP2.4 biological roles might be ethylene-dependent, we lack enough data to suggest or dismiss the notion that LjRAP2.4 positive regulation upon nodule organogenesis might be due to control of *L. japonicus* pathogenic response to *M. loti*; further research must be performed to address this issue. A key tool to investigate this point will be the analysis of the nodule formation phenotype in Lotus roots treated with the ethylene-precursor ACC and the ethylene inhibitor aminoethoxyvinylglycine (AVG). Overexpressing LjRAP2.4 plants and their wild-type counterparts will be subjected on the same nitrate conditions in which we observed this nodule phenotype, and threated with different concentrations of ACC or AVG; if this

phenotype is due to LjRAP2.4 involvement in the ethylene-induced nodule organogenesis regulatory pathway, overexpression of LjRAP2.4 is expected to compensate the negative regulation of ACC, attenuating the reduction of nodule numbers, and there will be not additive effect in nodule numbers in overexpressing LjRAP2.4 plants treated with AVG. Of course a parallel investigation has to be conducted on the RNAi plants showing about 60% silencing of LjRAP2.4 that should display a complementary phenotypes in the described conditions.

AtRAP2.4 was also reported as a factor playing a positive role in drought stress response, improving drought resistance by stomata regulation through an ABA-independent pathway (Lin et al. 2008). This phenotype is of particular interest in legumes because the efficiency of symbiotic nitrogen fixation is known to be strongly affected by drought conditions and legume plants overexpressing RAP2.4 could represent a valid genetic tool to overcome these inhibitory environmental conditions.

Bibliography

Allen, M. D., K. Yamasaki, et al. (1998). "A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA." EMBO J **17**(18): 5484-5496.

Almagro, A., S. H. Lin, et al. (2008). "Characterization of the Arabidopsis nitrate transporter NRT1.6 reveals a role of nitrate in early embryo development." Plant Cell **20**(12): 3289-3299.

Amor, B. B., S. L. Shaw, et al. (2003). "The NFP locus of Medicago truncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation." Plant J **34**(4): 495-506.

Andriankaja, A., A. Boisson-Dernier, et al. (2007). "AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif." Plant Cell **19**(9): 2866-2885.

Arrighi, J. F., A. Barre, et al. (2006). "The Medicago truncatula lysin [corrected] motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes." Plant Physiol **142**(1): 265-279.

Asamizu, E., Y. Shimoda, et al. (2008). "A positive regulatory role for LjERF1 in the nodulation process is revealed by systematic analysis of nodule-associated transcription factors of Lotus japonicus." Plant Physiol **147**(4): 2030-2040.

Aung, K., S. I. Lin, et al. (2006). "pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene." Plant Physiol **141**(3): 1000-1011.

Bagchi, R., M. Salehin, et al. (2012). "Functional assessment of the Medicago truncatula NIP/LATD protein demonstrates that it is a high-affinity nitrate transporter." Plant Physiol **160**(2): 906-916.

Baker, S. S., K. S. Wilhelm, et al. (1994). "The 5'-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression." Plant Mol Biol **24**(5): 701-713.

Banba, M., A. B. Siddique, et al. (2001). "Lotus japonicus forms early senescent root nodules with Rhizobium etli." Mol Plant Microbe Interact **14**(2): 173-180.

Barbulova, A., A. Rogato, et al. (2007). "Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in Lotus japonicus." Mol Plant Microbe Interact **20**(8): 994-1003.

Battisti, L., J. C. Lara, et al. (1992). "Specific oligosaccharide form of the Rhizobium meliloti exopolysaccharide promotes nodule invasion in alfalfa." Proc Natl Acad Sci U S A **89**(12): 5625-5629.

Binder, B. M., J. M. Walker, et al. (2007). "The Arabidopsis EIN3 binding F-Box proteins EBF1 and EBF2 have distinct but overlapping roles in ethylene signaling." Plant Cell **19**(2): 509-523.

Bisseling, T., C. Been, et al. (1983). "Nodule-specific host proteins in effective and ineffective root nodules of Pisum sativum." EMBO J **2**(6): 961-966.

Bisseling, T., W. van Staveren, et al. (1980). "The effect of waterlogging on the synthesis of the nitrogenase components in bacteroids of Rhizobium leguminosarum in root nodules of Pisum sativum." Biochem Biophys Res Commun **93**(3): 687-693.

Biswas, B., P. K. Chan, et al. (2009). "A novel ABA insensitive mutant of Lotus japonicus with a wilty phenotype displays unaltered nodulation regulation." Mol Plant **2**(3): 487-499.

Bright, L. J., Y. Liang, et al. (2005). "The LATD gene of Medicago truncatula is required for both nodule and root development." Mol Plant Microbe Interact **18**(6): 521-532.

Caba, J. M., M. L. Centeno, et al. (2000). "Inoculation and nitrate alter phytohormone levels in soybean roots: differences between a supernodulating mutant and the wild type." Planta **211**(1): 98-104.

Cabeza, R., B. Koester, et al. (2014). "An RNA Sequencing Transcriptome Analysis Reveals Novel Insights into Molecular Aspects of the Nitrate Impact on the Nodule Activity of Medicago truncatula." Plant Physiol **164**(1): 400-411.
Caetano-Anolles, G. and P. M. Gresshoff (1991). "Plant genetic control of nodulation." Annu Rev Microbiol **45**: 345-382.
Camanes, G., V. Pastor, et al. (2012). "A deletion in NRT2.1 attenuates Pseudomonas syringae-induced hormonal perturbation, resulting in primed plant defenses." Plant Physiol **158**(2): 1054-1066.
Cao, X. F., P. Linstead, et al. (1999). "Differential ethylene sensitivity of epidermal cells is involved in the establishment of cell pattern in the Arabidopsis root." Physiol Plant **106**(3): 311-317.
Cardenas, L., J. Dominguez, et al. (1995). "Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from Rhizobium etli." Plant Mol Biol **29**(3): 453-464.
Carroll, B. J. and A. Mathews (1990). Molecular biology of symbiotic nitrogen fixation. .
Castaings, L., A. Camargo, et al. (2009). "The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis." Plant J **57**(3): 426-435.
Charpentier, M., R. Bredemeier, et al. (2008). "Lotus japonicus CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis." Plant Cell **20**(12): 3467-3479.
Chen, C. Z., X. F. Lv, et al. (2012). "Arabidopsis NRT1.5 is another essential component in the regulation of nitrate reallocation and stress tolerance." Plant Physiol **159**(4): 1582-1590.
Chen, Y. F., N. Etheridge, et al. (2005). "Ethylene signal transduction." Ann Bot **95**(6): 901-915.
Chiang, C. S., G. Stacey, et al. (2004). "Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2." J Biol Chem **279**(29): 30150-30157.
Chiu, C. C., C. S. Lin, et al. (2004). "Mutation of a nitrate transporter, AtNRT1:4, results in a reduced petiole nitrate content and altered leaf development." Plant Cell Physiol **45**(9): 1139-1148.
Cho, M. J. and J. E. Harper (1991). "Effect of localized nitrate application on isoflavonoid concentration and nodulation in split-root systems of wild-type and nodulation-mutant soybean plants." Plant Physiol **95**(4): 1106-1112.
Chopin, F., M. Orsel, et al. (2007). "The Arabidopsis ATNRT2.7 nitrate transporter controls nitrate content in seeds." Plant Cell **19**(5): 1590-1602.
Christians, M. J., D. J. Gingerich, et al. (2009). "The BTB ubiquitin ligases ETO1, EOL1 and EOL2 act collectively to regulate ethylene biosynthesis in Arabidopsis by controlling type-2 ACC synthase levels." Plant J **57**(2): 332-345.
Clark, S. E., R. W. Williams, et al. (1997). "The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis." Cell **89**(4): 575-585.
Colebatch, G., G. Desbrosses, et al. (2004). "Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in Lotus japonicus." Plant J **39**(4): 487-512.
Cooper, J. B. and S. R. Long (1994). "Morphogenetic Rescue of Rhizobium meliloti Nodulation Mutants by trans-Zeatin Secretion." Plant Cell **6**(2): 215-225.
Criscuolo, G., V. T. Valkov, et al. (2012). "Molecular characterization of the Lotus japonicus NRT1(PTR) and NRT2 families." Plant Cell Environ **35**(9): 1567-1581.
Cullimore, J. V., R. Ranjeva, et al. (2001). "Perception of lipo-chitooligosaccharidic Nod factors in legumes." Trends Plant Sci **6**(1): 24-30.
Daniel, H., B. Spanier, et al. (2006). "From bacteria to man: archaic proton-dependent peptide transporters at work." Physiology (Bethesda) **21**: 93-102.
Das, S. K., R. R. Pathak, et al. (2007). "Genomewide computational analysis of nitrate response elements in rice and Arabidopsis." Mol Genet Genomics **278**(5): 519-525.
de Billy, F., C. Grosjean, et al. (2001). "Expression studies on AUX1-like genes in Medicago truncatula suggest that auxin is required at two steps in early nodule development." Mol Plant Microbe Interact **14**(3): 267-277.

Dechorgnat, J., O. Patrit, et al. (2012). "Characterization of the Nrt2.6 gene in Arabidopsis thaliana: a link with plant response to biotic and abiotic stress." PLoS One **7**(8): e42491.
Desbrosses, G. J. and J. Stougaard (2011). "Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways." Cell Host Microbe **10**(4): 348-358.
Ding, Y. and G. E. Oldroyd (2009). "Positioning the nodule, the hormone dictum." Plant Signal Behav **4**(2): 89-93.
Dixon, R. and D. Kahn (2004). "Genetic regulation of biological nitrogen fixation." Nat Rev Microbiol **2**(8): 621-631.
Dong, C. J. and J. Y. Liu (2010). "The Arabidopsis EAR-motif-containing protein RAP2.1 functions as an active transcriptional repressor to keep stress responses under tight control." BMC Plant Biol **10**: 47.
Eckardt, N. A. (2006). "Medicago truncatula CRE1 cytokinin receptor regulates nodulation and lateral root development." Plant Cell **18**(10): 2419.
Ehrhardt, D. W., R. Wais, et al. (1996). "Calcium spiking in plant root hairs responding to Rhizobium nodulation signals." Cell **85**(5): 673-681.
El Yahyaoui, F., H. Kuster, et al. (2004). "Expression profiling in Medicago truncatula identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program." Plant Physiol **136**(2): 3159-3176.
Fan, S. C., C. S. Lin, et al. (2009). "The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate." Plant Cell **21**(9): 2750-2761.
Fei, Y. J., Y. Kanai, et al. (1994). "Expression cloning of a mammalian proton-coupled oligopeptide transporter." Nature **368**(6471): 563-566.
Fischinger, S. A., J. J. Drevon, et al. (2006). "Nitrogen from senescing lower leaves of common bean is re-translocated to nodules and might be involved in a N-feedback regulation of nitrogen fixation." J Plant Physiol **163**(10): 987-995.
Fujikake, H., A. Yamazaki, et al. (2003). "Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules." J Exp Bot **54**(386): 1379-1388.
Fukao, T. and J. Bailey-Serres (2004). "Plant responses to hypoxia--is survival a balancing act?" Trends Plant Sci **9**(9): 449-456.
Geurts, R. and T. Bisseling (2002). "Rhizobium nod factor perception and signalling." Plant Cell **14 Suppl**: S239-249.
Gilmour, S. J., D. G. Zarka, et al. (1998). "Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression." Plant J **16**(4): 433-442.
Glass, A. D. and Z. Kotur (2013). "A reevaluation of the role of Arabidopsis NRT1.1 in high-affinity nitrate transport." Plant Physiol **163**(3): 1103-1106.
Glick, B. R. (2005). "Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase." FEMS Microbiol Lett **251**(1): 1-7.
Gonzalez-Rizzo, S., M. Crespi, et al. (2006). "The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti." Plant Cell **18**(10): 2680-2693.
Gonzalez, E. M., L. Galvez, et al. (2001). "Abscisic acid induces a decline in nitrogen fixation that involves leghaemoglobin, but is independent of sucrose synthase activity." J Exp Bot **52**(355): 285-293.
Gresshoff, P. M., D. Lohar, et al. (2009). "Genetic analysis of ethylene regulation of legume nodulation." Plant Signal Behav **4**(9): 818-823.
Groth, M., N. Takeda, et al. (2010). "NENA, a Lotus japonicus homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development." Plant Cell **22**(7): 2509-2526.

Gruber, V., S. Blanchet, et al. (2009). "Identification of transcription factors involved in root apex responses to salt stress in Medicago truncatula." Mol Genet Genomics **281**(1): 55-66.
Guo, F. Q., R. Wang, et al. (2001). "The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is activated and functions in nascent organ development during vegetative and reproductive growth." Plant Cell **13**(8): 1761-1777.
Guo, F. Q., R. Wang, et al. (2002). "The Arabidopsis dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is regulated by auxin in both shoots and roots." J Exp Bot **53**(370): 835-844.
Guzman, P. and J. R. Ecker (1990). "Exploiting the triple response of Arabidopsis to identify ethylene-related mutants." Plant Cell **2**(6): 513-523.
Hannah, M. A., D. Wiese, et al. (2006). "Natural genetic variation of freezing tolerance in Arabidopsis." Plant Physiol **142**(1): 98-112.
Hattori, Y., K. Nagai, et al. (2009). "The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water." Nature **460**(7258): 1026-1030.
Hauser, M., V. Narita, et al. (2001). "Multiplicity and regulation of genes encoding peptide transporters in Saccharomyces cerevisiae." Mol Membr Biol **18**(1): 105-112.
Heidstra, R., W. C. Yang, et al. (1997). "Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction." Development **124**(9): 1781-1787.
Hinz, M., I. W. Wilson, et al. (2010). "Arabidopsis RAP2.2: an ethylene response transcription factor that is important for hypoxia survival." Plant Physiol **153**(2): 757-772.
Hirsch, A. M. and Y. Fang (1994). "Plant hormones and nodulation: what's the connection?" Plant Mol Biol **26**(1): 5-9.
Hirsch, A. M. and C. A. Smith (1987). "Effects of Rhizobium meliloti nif and fix mutants on alfalfa root nodule development." J Bacteriol **169**(3): 1137-1146.
Hogslund, N., S. Radutoiu, et al. (2009). "Dissection of symbiosis and organ development by integrated transcriptome analysis of lotus japonicus mutant and wild-type plants." PLoS One **4**(8): e6556.
Horchani, F., M. Prevot, et al. (2011). "Both plant and bacterial nitrate reductases contribute to nitric oxide production in Medicago truncatula nitrogen-fixing nodules." Plant Physiol **155**(2): 1023-1036.
Hsu, P. K. and Y. F. Tsay (2013). "Two phloem nitrate transporters, NRT1.11 and NRT1.12, are important for redistributing xylem-borne nitrate to enhance plant growth." Plant Physiol **163**(2): 844-856.
Huang, N. C., K. H. Liu, et al. (1999). "Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake." Plant Cell **11**(8): 1381-1392.
Hunter, W. J. (1993). "Ethylene Production by Root Nodules and Effect of Ethylene on Nodulation in Glycine max." Appl Environ Microbiol **59**(6): 1947-1950.
Jeudy, C., S. Ruffel, et al. (2010). "Adaptation of Medicago truncatula to nitrogen limitation is modulated via local and systemic nodule developmental responses." New Phytol **185**(3): 817-828.
Jiang, C., B. Iu, et al. (1996). "Requirement of a CCGAC cis-acting element for cold induction of the BN115 gene from winter Brassica napus." Plant Mol Biol **30**(3): 679-684.
Jofuku, K. D., B. G. den Boer, et al. (1994). "Control of Arabidopsis flower and seed development by the homeotic gene APETALA2." Plant Cell **6**(9): 1211-1225.
Jung, K. H., Y. S. Seo, et al. (2010). "The submergence tolerance regulator Sub1A mediates stress-responsive expression of AP2/ERF transcription factors." Plant Physiol **152**(3): 1674-1692.
Kanayama, Y. and Y. Yamamoto (1990). "Inhibition of nitrogen fixation in soybean plants supplied with nitrate II. Accumulation and properties of nitrosylleghemoglobin in nodules." Plant and Cell Physiology **31**(2): 207-214.

Kanno, Y., A. Hanada, et al. (2012). "Identification of an abscisic acid transporter by functional screening using the receptor complex as a sensor." Proc Natl Acad Sci U S A **109**(24): 9653-9658.
Karaba, A., S. Dixit, et al. (2007). "Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene." Proc Natl Acad Sci U S A **104**(39): 15270-15275.
Karas, B., J. Murray, et al. (2005). "Invasion of Lotus japonicus root hairless 1 by Mesorhizobium loti involves the nodulation factor-dependent induction of root hairs." Plant Physiol **137**(4): 1331-1344.
Karim, S., K. O. Holmstrom, et al. (2007). "AtPTR3, a wound-induced peptide transporter needed for defence against virulent bacterial pathogens in Arabidopsis." Planta **225**(6): 1431-1445.
Karim, S., D. Lundh, et al. (2005). "Structural and functional characterization of AtPTR3, a stress-induced peptide transporter of Arabidopsis." J Mol Model **11**(3): 226-236.
Kato, K., K. Kanahama, et al. (2010). "Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in Lotus root nodules." J Plant Physiol **167**(3): 238-241.
Kiba, T., A. B. Feria-Bourrellier, et al. (2012). "The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants." Plant Cell **24**(1): 245-258.
Komarova, N. Y., K. Thor, et al. (2008). "AtPTR1 and AtPTR5 transport dipeptides in planta." Plant Physiol **148**(2): 856-869.
Kouchi, H., K. Shimomura, et al. (2004). "Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, Lotus japonicus." DNA Res **11**(4): 263-274.
Koussevitzky, S., A. Nott, et al. (2007). "Signals from chloroplasts converge to regulate nuclear gene expression." Science **316**(5825): 715-719.
Krouk, G., N. M. Crawford, et al. (2010). "Nitrate signaling: adaptation to fluctuating environments." Curr Opin Plant Biol **13**(3): 266-273.
Krouk, G., B. Lacombe, et al. (2010). "Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants." Dev Cell **18**(6): 927-937.
Krusell, L., L. H. Madsen, et al. (2002). "Shoot control of root development and nodulation is mediated by a receptor-like kinase." Nature **420**(6914): 422-426.
Lee, K. H. and T. A. Larue (1992). "Ethylene as a Possible Mediator of Light- and Nitrate-Induced Inhibition of Nodulation of Pisum sativum L. cv Sparkle." Plant Physiol **100**(3): 1334-1338.
Lee, K. H. and T. A. Larue (1992). "Exogenous Ethylene Inhibits Nodulation of Pisum sativum L. cv Sparkle." Plant Physiol **100**(4): 1759-1763.
Leran, S., K. Varala, et al. (2014). "A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants." Trends Plant Sci **19**(1): 5-9.
Li, J. Y., Y. L. Fu, et al. (2010). "The Arabidopsis nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance." Plant Cell **22**(5): 1633-1646.
Li, W., Y. Wang, et al. (2007). "Dissection of the AtNRT2.1:AtNRT2.2 inducible high-affinity nitrate transporter gene cluster." Plant Physiol **143**(1): 425-433.
Lievens, S., S. Goormachtig, et al. (2005). "Gibberellins are involved in nodulation of Sesbania rostrata." Plant Physiol **139**(3): 1366-1379.
Lin, R. C., H. J. Park, et al. (2008). "Role of Arabidopsis RAP2.4 in regulating light- and ethylene-mediated developmental processes and drought stress tolerance." Mol Plant **1**(1): 42-57.
Lin, S. H., H. F. Kuo, et al. (2008). "Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport." Plant Cell **20**(9): 2514-2528.
Little, D. Y., H. Rao, et al. (2005). "The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues." Proc Natl Acad Sci U S A **102**(38): 13693-13698.
Liu, K. H., C. Y. Huang, et al. (1999). "CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake." Plant Cell **11**(5): 865-874.

Liu, K. H. and Y. F. Tsay (2003). "Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation." EMBO J **22**(5): 1005-1013.
Liu, Q., M. Kasuga, et al. (1998). "Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis." Plant Cell **10**(8): 1391-1406.
Lohar, D., J. Stiller, et al. (2009). "Ethylene insensitivity conferred by a mutated Arabidopsis ethylene receptor gene alters nodulation in transgenic Lotus japonicus." Ann Bot **104**(2): 277-285.
Lohar, D. P., J. E. Schaff, et al. (2004). "Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses." Plant J **38**(2): 203-214.
Lohar, D. P., N. Sharopova, et al. (2006). "Transcript analysis of early nodulation events in Medicago truncatula." Plant Physiol **140**(1): 221-234.
Lorenzo, O., R. Piqueras, et al. (2003). "ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense." Plant Cell **15**(1): 165-178.
Lorteau, M. A., B. J. Ferguson, et al. (2001). "Effects of cytokinin on ethylene production and nodulation in pea (Pisum sativum) cv. Sparkle." Physiol Plant **112**(3): 421-428.
Ma, W., F. C. Guinel, et al. (2003). "Rhizobium leguminosarum biovar viciae 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants." Appl Environ Microbiol **69**(8): 4396-4402.
Madsen, E. B., M. Antolin-Llovera, et al. (2011). "Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5." Plant J **65**(3): 404-417.
Madsen, L. H., L. Tirichine, et al. (2010). "The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus." Nat Commun **1**: 10.
Magnani, E., K. Sjolander, et al. (2004). "From endonucleases to transcription factors: evolution of the AP2 DNA binding domain in plants." Plant Cell **16**(9): 2265-2277.
Mantiri, F. R., S. Kurdyukov, et al. (2008). "The transcription factor MtSERF1 of the ERF subfamily identified by transcriptional profiling is required for somatic embryogenesis induced by auxin plus cytokinin in Medicago truncatula." Plant Physiol **146**(4): 1622-1636.
Maruyama, K., Y. Sakuma, et al. (2004). "Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems." Plant J **38**(6): 982-993.
Matamoros, M. A., L. M. Baird, et al. (1999). "Stress-induced legume root nodule senescence. Physiological, biochemical, and structural alterations." Plant Physiol **121**(1): 97-112.
Matsukura, S., J. Mizoi, et al. (2010). "Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes." Mol Genet Genomics **283**(2): 185-196.
Mattsson, J., Z. R. Sung, et al. (1999). "Responses of plant vascular systems to auxin transport inhibition." Development **126**(13): 2979-2991.
Meilhoc, E., A. Boscari, et al. (2011). "Nitric oxide in legume-rhizobium symbiosis." Plant Sci **181**(5): 573-581.
Meredith, D. (2009). "Review. The mammalian proton-coupled peptide cotransporter PepT1: sitting on the transporter-channel fence?" Philos Trans R Soc Lond B Biol Sci **364**(1514): 203-207.
Miller, A., F. Xiaorong, et al. (2007). "Nitrate transport and signalling." Journal of Experimental Botany **58**(9): 10p.
Mizoi, J., K. Shinozaki, et al. (2012). "AP2/ERF family transcription factors in plant abiotic stress responses." Biochim Biophys Acta **1819**(2): 86-96.
Morere-Le Paven, M. C., L. Viau, et al. (2011). "Characterization of a dual-affinity nitrate transporter MtNRT1.3 in the model legume Medicago truncatula." J Exp Bot **62**(15): 5595-5605.
Mortier, V., G. Den Herder, et al. (2010). "CLE peptides control Medicago truncatula nodulation locally and systemically." Plant Physiol **153**(1): 222-237.

Murray, J. D., B. J. Karas, et al. (2007). "A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis." Science **315**(5808): 101-104.
Nakano, T., K. Suzuki, et al. (2006). "Genome-wide analysis of the ERF gene family in Arabidopsis and rice." Plant Physiol **140**(2): 411-432.
Naudin, C., G. Corre-Hellou, et al. (2011). "Inhibition and recovery of symbiotic N2 fixation by peas (Pisum sativum L.) in response to short-term nitrate exposure." Plant and Soil **346**(1-2): 275-287.
Neill, S., R. Barros, et al. (2008). "Nitric oxide, stomatal closure, and abiotic stress." J Exp Bot **59**(2): 165-176.
Nelson, D. L. and M. M. Cox (2008). Lehninger Principles of Biochemistry.
Newstead, S., D. Drew, et al. (2011). "Crystal structure of a prokaryotic homologue of the mammalian oligopeptide-proton symporters, PepT1 and PepT2." EMBO J **30**(2): 417-426.
Nishimura, C., Y. Ohashi, et al. (2004). "Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis." Plant Cell **16**(6): 1365-1377.
Nishimura, R., M. Ohmori, et al. (2002). "The novel symbiotic phenotype of enhanced-nodulating mutant of Lotus japonicus: astray mutant is an early nodulating mutant with wider nodulation zone." Plant Cell Physiol **43**(8): 853-859.
Nour-Eldin, H. H., T. G. Andersen, et al. (2012). "NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds." Nature **488**(7412): 531-534.
Nukui, N., H. Ezura, et al. (2004). "Transgenic Lotus japonicus with an ethylene receptor gene Cm-ERS1/H70A enhances formation of infection threads and nodule primordia." Plant Cell Physiol **45**(4): 427-435.
Ohme-Takagi, M. and H. Shinshi (1995). "Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element." Plant Cell **7**(2): 173-182.
Oka-Kira, E. and M. Kawaguchi (2006). "Long-distance signaling to control root nodule number." Curr Opin Plant Biol **9**(5): 496-502.
Okamoto, M., J. J. Vidmar, et al. (2003). "Regulation of NRT1 and NRT2 gene families of Arabidopsis thaliana: responses to nitrate provision." Plant Cell Physiol **44**(3): 304-317.
Okamoto, S., E. Ohnishi, et al. (2009). "Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation." Plant Cell Physiol **50**(1): 67-77.
Oldroyd, G. E., E. M. Engstrom, et al. (2001). "Ethylene inhibits the Nod factor signal transduction pathway of Medicago truncatula." Plant Cell **13**(8): 1835-1849.
Omrane, S. and M. Chiurazzi (2009). "A variety of regulatory mechanisms are involved in the nitrogen-dependent modulation of the nodule organogenesis program in legume roots." Plant Signal Behav **4**(11): 1066-1068.
Omrane, S., A. Ferrarini, et al. (2009). "Symbiotic competence in Lotus japonicus is affected by plant nitrogen status: transcriptomic identification of genes affected by a new signalling pathway." New Phytol **183**(2): 380-394.
Ortega-Martinez, O., M. Pernas, et al. (2007). "Ethylene modulates stem cell division in the Arabidopsis thaliana root." Science **317**(5837): 507-510.
Pacios-Bras, C., H. R. Schlaman, et al. (2003). "Auxin distribution in Lotus japonicus during root nodule development." Plant Mol Biol **52**(6): 1169-1180.
Pasternak, T. P., E. Prinsen, et al. (2002). "The Role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa." Plant Physiol **129**(4): 1807-1819.
Patriarca, E. J., R. Tate, et al. (2004). "Organogenesis of legume root nodules." Int Rev Cytol **234**: 201-262.
Patriarca, E. J., R. Tate, et al. (2002). "Key role of bacterial NH(4)(+) metabolism in Rhizobium-plant symbiosis." Microbiol Mol Biol Rev **66**(2): 203-222.

Peng, H. P., T. Y. Lin, et al. (2005). "Differential expression of genes encoding 1-aminocyclopropane-1-carboxylate synthase in Arabidopsis during hypoxia." Plant Mol Biol **58**(1): 15-25.
Penmetsa, R. V., P. Uribe, et al. (2008). "The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations." Plant J **55**(4): 580-595.
Prasad, M. E., A. Schofield, et al. (2010). "Arabidopsis RING E3 ligase XBAT32 regulates lateral root production through its role in ethylene biosynthesis." Plant Physiol **153**(4): 1587-1596.
Price, J., A. Laxmi, et al. (2004). "Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis." Plant Cell **16**(8): 2128-2150.
Qiao, H., K. N. Chang, et al. (2009). "Interplay between ethylene, ETP1/ETP2 F-box proteins, and degradation of EIN2 triggers ethylene responses in Arabidopsis." Genes Dev **23**(4): 512-521.
Rae, L., N. T. Lao, et al. (2011). "Regulation of multiple aquaporin genes in Arabidopsis by a pair of recently duplicated DREB transcription factors." Planta **234**(3): 429-444.
Reid, D. E., B. J. Ferguson, et al. (2011). "Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation." Mol Plant Microbe Interact **24**(5): 606-618.
Reyes, J. C., M. I. Muro-Pastor, et al. (2004). "The GATA family of transcription factors in Arabidopsis and rice." Plant Physiol **134**(4): 1718-1732.
Riechmann, J. L. and E. M. Meyerowitz (1998). "The AP2/EREBP family of plant transcription factors." Biol Chem **379**(6): 633-646.
Rubin, G., T. Tohge, et al. (2009). "Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis." Plant Cell **21**(11): 3567-3584.
Ruffel, S., S. Freixes, et al. (2008). "Systemic signaling of the plant nitrogen status triggers specific transcriptome responses depending on the nitrogen source in Medicago truncatula." Plant Physiol **146**(4): 2020-2035.
Sakuma, Y., Q. Liu, et al. (2002). "DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression." Biochem Biophys Res Commun **290**(3): 998-1009.
Sakuma, Y., K. Maruyama, et al. (2006). "Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression." Plant Cell **18**(5): 1292-1309.
Sandal, N., L. Krusell, et al. (2002). "A genetic linkage map of the model legume Lotus japonicus and strategies for fast mapping of new loci." Genetics **161**(4): 1673-1683.
Sato, S., Y. Nakamura, et al. (2008). "Genome structure of the legume, Lotus japonicus." DNA Res **15**(4): 227-239.
Schauser, L., A. Roussis, et al. (1999). "A plant regulator controlling development of symbiotic root nodules." Nature **402**(6758): 191-195.
Scheible, W. R., R. Morcuende, et al. (2004). "Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen." Plant Physiol **136**(1): 2483-2499.
Schmidt, J. S., J. E. Harper, et al. (1999). "Regulation of soybean nodulation independent of ethylene signaling." Plant Physiol **119**(3): 951-960.
Segonzac, C., J. C. Boyer, et al. (2007). "Nitrate efflux at the root plasma membrane: identification of an Arabidopsis excretion transporter." Plant Cell **19**(11): 3760-3777.
Sharoni, A. M., M. Nuruzzaman, et al. (2011). "Gene structures, classification and expression models of the AP2/EREBP transcription factor family in rice." Plant Cell Physiol **52**(2): 344-360.
Shigyo, M., M. Hasebe, et al. (2006). "Molecular evolution of the AP2 subfamily." Gene **366**(2): 256-265.
Solano, R., A. Stepanova, et al. (1998). "Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1." Genes Dev **12**(23): 3703-3714.

Solcan, N., J. Kwok, et al. (2012). "Alternating access mechanism in the POT family of oligopeptide transporters." EMBO J **31**(16): 3411-3421.
Spaink, H. P. (2000). "Root nodulation and infection factors produced by rhizobial bacteria." Annu Rev Microbiol **54**: 257-288.
Spaink, H. P., D. M. Sheeley, et al. (1991). "A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium." Nature **354**(6349): 125-130.
Stracke, S., C. Kistner, et al. (2002). "A plant receptor-like kinase required for both bacterial and fungal symbiosis." Nature **417**(6892): 959-962.
Suzuki, A., M. Akune, et al. (2004). "Control of nodule number by the phytohormone abscisic Acid in the roots of two leguminous species." Plant Cell Physiol **45**(7): 914-922.
Takanashi, K., H. Takahashi, et al. (2012). "Tissue-specific transcriptome analysis in nodules of Lotus japonicus." Mol Plant Microbe Interact **25**(7): 869-876.
Tanimoto, M., K. Roberts, et al. (1995). "Ethylene is a positive regulator of root hair development in Arabidopsis thaliana." Plant J **8**(6): 943-948.
Tirichine, L., E. K. James, et al. (2006). "Spontaneous root-nodule formation in the model legume Lotus japonicus: a novel class of mutants nodulates in the absence of rhizobia." Mol Plant Microbe Interact **19**(4): 373-382.
Tominaga, A., M. Nagata, et al. (2010). "Effect of abscisic acid on symbiotic nitrogen fixation activity in the root nodules of Lotus japonicus." Plant Signal Behav **5**(4): 440-443.
Tsay, Y. F., C. C. Chiu, et al. (2007). "Nitrate transporters and peptide transporters." FEBS Lett **581**(12): 2290-2300.
Tsay, Y. F., J. I. Schroeder, et al. (1993). "The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter." Cell **72**(5): 705-713.
Tsuchisaka, A., G. Yu, et al. (2009). "A combinatorial interplay among the 1-aminocyclopropane-1-carboxylate isoforms regulates ethylene biosynthesis in Arabidopsis thaliana." Genetics **183**(3): 979-1003.
Uchiumi, T., T. Ohwada, et al. (2004). "Expression islands clustered on the symbiosis island of the Mesorhizobium loti genome." J Bacteriol **186**(8): 2439-2448.
Unkles, S. E., K. L. Hawker, et al. (1991). "crnA encodes a nitrate transporter in Aspergillus nidulans." Proc Natl Acad Sci U S A **88**(1): 204-208.
Urbanski, D. F., A. Malolepszy, et al. (2012). "Genome-wide LORE1 retrotransposon mutagenesis and high-throughput insertion detection in Lotus japonicus." Plant J **69**(4): 731-741.
Vasse, J., F. de Billy, et al. (1990). "Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules." J Bacteriol **172**(8): 4295-4306.
Vogel, J. P., K. E. Woeste, et al. (1998). "Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of Arabidopsis confer cytokinin insensitivity and ethylene overproduction, respectively." Proc Natl Acad Sci U S A **95**(8): 4766-4771.
Walch-Liu, P., S. Filleur, et al. (2005). "Signaling mechanisms integrating root and shoot responses to changes in the nitrogen supply." Photosynth Res **83**(2): 239-250.
Wang, G. and M. Fiers (2010). "CLE peptide signaling during plant development." Protoplasma **240**(1-4): 33-43.
Wang, R., K. Guegler, et al. (2000). "Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate." Plant Cell **12**(8): 1491-1509.
Wang, R., D. Liu, et al. (1998). "The Arabidopsis CHL1 protein plays a major role in high-affinity nitrate uptake." Proc Natl Acad Sci U S A **95**(25): 15134-15139.
Wang, X., Y. Bian, et al. (2012). "A comprehensive differential proteomic study of nitrate deprivation in Arabidopsis reveals complex regulatory networks of plant nitrogen responses." J Proteome Res **11**(4): 2301-2315.

Wang, Y. Y. and Y. F. Tsay (2011). "Arabidopsis nitrate transporter NRT1.9 is important in phloem nitrate transport." Plant Cell **23**(5): 1945-1957.
Weichert, A., C. Brinkmann, et al. (2012). "AtPTR4 and AtPTR6 are differentially expressed, tonoplast-localized members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family." Planta **235**(2): 311-323.
Weigel, D. (1995). "The APETALA2 domain is related to a novel type of DNA binding domain." Plant Cell **7**(4): 388-389.
Wilson, I. D., S. J. Neill, et al. (2008). "Nitric oxide synthesis and signalling in plants." Plant Cell Environ **31**(5): 622-631.
Yamaguchi-Shinozaki, K. and K. Shinozaki (1992). "A novel Arabidopsis DNA binding protein contains the conserved motif of HMG-box proteins." Nucleic Acids Res **20**(24): 6737.
Yamaguchi-Shinozaki, K. and K. Shinozaki (1993). "Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants." Mol Gen Genet **236**(2-3): 331-340.
Yan, D. (2007). "Protection of the glutamate pool concentration in enteric bacteria." Proc Natl Acad Sci U S A **104**(22): 9475-9480.
Yano, K., S. Yoshida, et al. (2008). "CYCLOPS, a mediator of symbiotic intracellular accommodation." Proc Natl Acad Sci U S A **105**(51): 20540-20545.
Zahran, H. H. (1999). "Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate." Microbiol Mol Biol Rev **63**(4): 968-989, table of contents.
Zhang, H. and B. G. Forde (1998). "An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture." Science **279**(5349): 407-409.

Index of Abbreviations

ABA – abscisic acid

ABC – auxin-burst control

ABR – ABA-responsive element

ACC - 1-aminocyclopropanme-1-carboxylic acid

accD - 1-aminocyclopropanme-1-carboxylic acid deaminase

ADP – adenoside diphosfate

ATP – adenosine triphosfate

BLAST - Basic Local Alignment Search Tool

BP – brassinosteroid

BTBT - *Broad complex/Tramtrack/Brick-a-Brack*

CaMV - *Cauliflower Mosaic Virus*

CARB - Centre for Carbohydrate Recognition and Signalling

CIM – Callous Induction Medium

cDNA - *complementary DNA*

Cef - Cefotaxime

cRNA – *complementary RNA*

CTR – C-repeat

CZ – central zone

DEPC - Diethylpyrocarbonate

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide Triphosphates

DRE - cis-acting dehydration- responsive element

EDTA - ethylenediaminetetraacetic acid

EST - *expressed sequence tag*

EREBP - ethylene-responsive element binding protein

GA – gibberellic acid

GDH - glutamate desidrogenase

Gln - Glutamine

Glu – Glutamate

GOGAT - glutamine oxoglutarate aminotransferase

GS - glutamine synthetase

HATS – high-affinity transport system

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hpt – hygromycin resistance cassette

Hyg - hygromycin

hygr- hygromycin resistance cassette

IAA – indole-3-acetic acid

JA – Jasmonic acid

LB - Luria-Bertani

LB – *Left Border*

LATS – low-affinity transport system

LEA – late embryogenesis abundant

LTRE – low-temperature responsive element

LORE1 – Lotus retrotransposson one

MES - 2.4-Dichlorophenoxyacetic acid

mRNA – *messenger RNA*

MS - Murashige & Skoog

NADPH - nicotinamide adenine dinucleotide phosphate

NiR – nitrite reductase

NR – nitrate reductase

NRE – nitrate responsive elements

NPA - 1-N-aphtylphthalamic acid

NPF – nitrate transporter family

OD – *Optical Density*

PBS - *Phosphate buffered saline*

PCR – *Polymerase Chain Reaction*

PepT/PTR – peptide transprorter

Pi – inorganic phosphate

PL – polylinker

PTR – peptide transporters

POT – Proton-coupled Oligopeptide transporter

PZ – peripheral zones

RB – *Right Border*

RIM – Root Induction Medium

RNA - ribonucleic acid

RNAi – *RNA interference*

SA – salycillic acid

SAM – shoot apical meristem

SD – *standard deviation*

SEM – Shoot Elongation Medium

SIM – Shoot Induction Medium

SOC – *Super Optimal Broth*

SL15 – Solute carrier 15

TAE - Tris Acetate EDTA

T-DNA – transfer DNA

TDZ – Thidiazuron

TE - Tris EDTA

TF – transcription factor

TIBA - tri-indobenzoic acid

Tm – melting temperature